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EDILZA SILVA DO NASCIMENTO

INHAME (*Dioscorea cayennensis*) CULTIVADO NA PARAÍBA: CARACTERIZAÇÃO E PRODUÇÃO DE CONCENTRADO PROTEICO PARA PROSPECÇÃO DE PEPTÍDEOS BIOATIVOS OBTIDOS ATRAVÉS DE DIGESTÃO SIMULADA *IN VITRO*

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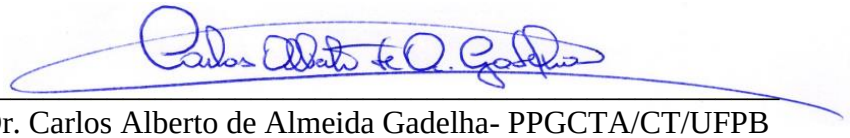

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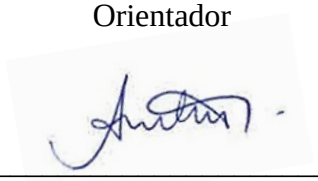

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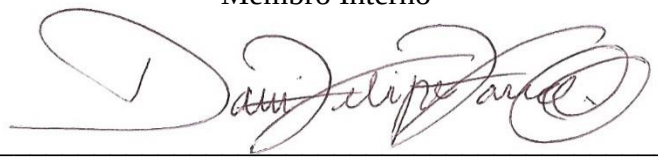

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"Todas as coisas contribuem para o bem dos que amam a Deus".

Apóstolo Paulo

(Carta aos Romanos 8.28)

RESUMO

O tubérculo inhame tem importância na cultura alimentar do Nordeste, além de ser considerado um alimento funcional, que apresenta proteínas com potenciais propriedades bioativas. Objetivou-se com este estudo caracterizar o perfil peptídico e realizar a bioprospecção de peptídeos bioativos obtidos da digestão gastrointestinal simulada *in vitro* das proteínas do inhame (*Dioscorea cayennensis)* cultivado no Nordeste do Brasil. Para tanto, inicialmente, a amostra obtida foi analisada quanto a composição química parcial, seguida da obtenção da farinha para o preparo do extrato e concentrado proteico de inhame (CPI), submetido à digestão simulada *in vitro*, por fim caracterização e perfil dos peptídeos obtidos. O extrato proteico foi submetido à precipitação isoelétrica para obtenção do CPI, que em seguida foi dialisado, congelado e liofilizado. O CPI foi submetido à digestão gastrointestinal (GI) simulada, gerando duas fases: hidrolisado fase gástrica (HFG) e hidrólise contínua até o hidrolisado fase gastrointestinal (HFGI). As amostras: CPI, HFG e HFGI foram submetidas às análises de perfil proteico/peptídico: aminoácidos totais e livres (AAL), massa molecular (MM), hidrofobicidade por cromatografia em fase reversa e eletroforese (SDS-Tricina); perfil proteico (eletroforese-2D) e perfil peptídico. Os peptídeos identificados foram submetidos à predição de bioatividade através da Database of bioactive peptides (BIOPEP) e PeptideRanker (atividade inibitória da enzima conversora de angiotensina-ECA). Para validar os resultados obtidos *in silico*, foram realizadas análises de determinação de atividades biológicas *in vitro*: antioxidante (DPPH, ABTS, ORAC e proteção dos danos ao DNA), antibacteriana e inibidora da ECA. Os resultados da eletroforese 2D evidenciaram a presença da proteína dioscorina, como sendo a proteína majoritária do inhame. O CPI apresentou 64,0 % de proteínas, contendo todos os aminoácidos essenciais com escore adequado de acordo com a FAO, exceto os aminoácidos sulfurados. Após a digestão GI do CPI, foi evidenciada aumento da liberação significativa ($p < 0,5$) de AAL. A eficiência da digestão GI foi verificada conforme perfil de distribuição de MM, com aproximadamente 71,0 % de peptídeos com MM <2 kDa gerados no HFGI. Os peptídeos identificados, em sua maioria são pertencentes à proteína dioscorina de *D. cayennensis.* Peptídeos do HFG tiveram sua MM diminuida com a progressão da hidrólise GI, resultando em peptídeos de menor MM no HFGI**.** Ademais, identificou-se sequências peptídicas com potenciais para peptídeos bioativos, com atividade anti-hipertensiva, antidiabética, antioxidante e antibacteriana a partir da predição por BIOPEP. Verificou-se nos HFG e HFGI a presença de sequência peptídica contendo prolina e leucina (LA**PLPL**) que pode contribuir para a inibição da ECA, assim como peptídeos contendo resíduos aromáticos no C-terminal (DIT**WT** e **F**LS**WT**) que possibilitam a doação de elétrons e estabilização de radicais livres. Esses achados sugerem que essas características dos peptídeos contribuíram para a atividade antioxidante em todos os ensaios *in vitro*. Os peptídeos hidrofóbicos e com carga positiva (IFDQTLG**KLR**) podem ter possibilitado a inibição do crescimento da bactéria *Escherichia coli*. Portanto, os peptídeos obtidos após a digestão GI das proteínas do inhame podem contribuir na prevenção do desenvolvimento de infecções e doenças crônicas degenerativas, com impacto para a saúde humana.

PALAVRAS-CHAVE: proteína vegetal, peptídeos antibacterianos, peptídeos antioxidantes, peptídeos anti-hipertensivos, BIOPEP.

ABSTRACT

The yam is an important tuber in Brazil Northeast food culture, also being considered a functional food, as it has proteins with potential bioactive properties. The objective of this study was to characterize the peptide profile and perform the bioprospection of bioactive peptides obtained from *in vitro* simulated gastrointestinal digestion of yam proteins (*Dioscorea cayennensis*) grown in Brazil. Therefore, the sample obtained was initially analyzed for partial chemical composition, followed by obtaining the flour for the yam extract and protein concentrate (YPC) preparation, submitted to simulated digestion in vitro, and finally the characterization and profile of the obtained peptides were performed. The protein extract was subjected to isoelectric precipitation to obtain the YPC, which was then dialyzed and freeze-dried. The YPC was submitted to simulated gastrointestinal (GI) digestion, generating two phases: the gastric phase hydrolysate (GPH) and continuous hydrolysis until the gastrointestinal phase hydrolysate (GIPH). The samples: YPC, GPH and GIPH were submitted to analysis of protein and peptide profile: total amino acids and free amino acids (FAA); molecular mass (MM); hydrophobicity by reverse phase chromatography and electrophoresis (Tricine-SDS-PAGE); protein profile (two-dimensional gel electrophoresis) and peptide profile (nano-LC-ESI-MS/MS, MALDI-TOF). The identified peptides were submitted to the bioactivity prediction through the Bioactive Peptides Database (BIOPEP) and PeptideRanker (inhibitory activity of the angiotensin-converting enzyme-ACE). To validate results obtained *in silico*, analysis were performed to determine biological activities *in vitro*: antioxidant (DPPH, ABTS, ORAC and protection from DNA damage), antibacterial and ACE's inhibitor. The results of 2D-electrophoresis show the presence of the dioscorin protein, as the yam's major protein. The YCP resulted in 64.0 % protein, with all essential amino acids with an appropriate score according to FAO, except sulfur amino acids. After the GI digestion of the YPC, a significant increase in the release ($p < 0.5$) of AAL was evidenced. The efficiency of GI digestion was verified according to the distribution profile of MW, with approximately 71.0 % of peptides with MW <2 kDa generated in the GIPH. Most of the identified peptides belong to the *D. cayennensis* dioscorin protein. GPH peptides had their MM decreased with the progression of GI hydrolysis, resulting in smaller MM peptides in GIPH. In addition, sequences with potential for bioactive peptides, with antihypertensive, antidiabetic, antioxidant and antibacterial activity were identified from the BIOPEP prediction. The presence of a peptide sequence containing proline and leucine (LA**PLPL**) was found in the GPH and GIPH, which can contribute to the inhibition of ACE, as well as peptides containing aromatic residues in the C-terminal (DIT**WT** and **F**LS**WT**) that allow the donation of electrons and stabilization of free radicals. These findings suggest that these characteristics contributed to the antioxidant activity in all *in vitro* assays. Hydrophobic and positively charged peptides (IFDQTLG**KLR**) may have made it possible to inhibit the growth of the bacteria *Escherichia coli*. Therefore, the peptides obtained after the GI digestion of yam proteins can contribute to the prevention of infections and chronic degenerative diseases development, with an impact on human health.

KEYWORDS: tuber protein, antibacterial peptides, antioxidant peptides, antihypertensive peptides, BIOPEP.

LISTA DE FIGURAS

ARTIGO I

ARTIGO II

LISTA DE TABELAS

ARTIGO I

ARTIGO II

LISTRA DE QUADROS

LISTA DE ABREVIATURAS E SIGLAS

AA aminoácido

AAL aminoácidos livres

ABTS●+ Radical 2,2-azino-bis (3-etilbezotiazolina)-6-ácido sulfônico

ACE- Angiotensin converting enzyme

ANOVA Análise de Variância

AOAC Association of Official Analytical Chemist methods

AUC area under the curve

BIOPEP Database of Bioactive Peptides

bs. Base seca

CLAE-FR cromatografia líquida de alta eficiência em fase reversa

ºC Graus Celsius

Da Dalton

DH Degree of hydrolysis

DPP-IV Dipeptidil peptidase IV

DPPH●+ Radical 2,2-difenil-1picril-hidrazila

DREB- Dehydration-responsive element-binding

DTT Dithiothreitol

ECA- Enzima conversora de angiotensina

FAO Food and Agriculture Organization

FPLC Fast protein liquid chromatography

WHO World Health Organization

FAOSTAT Food and Agriculture Organization Corporate Statistical Database

g Grama

h Hora

HCl Ácido clorídrico

ITAL Instituto de Tecnologia dos Alimentos

kDa Kilodaltons

MALDI-TOF Matrix Associated Laser Desorption-Ionization – Time of Flight

mM Milimolar

mm Milímetro

mA Miliampère

mg Miligramas

μg Micrograma

μL Microlitro

μ Micromol

min Minutos

nanoLC-ESI-MS/MS nanoflux liquid chromatography coupled to sequential mass spectrometry with Electrospray Ionization

NAOH Hidróxido de sódio

nº Número

nm Nanômetros

nmol Nanomol

OPA o-phthaldialdehyde

OMS Organização mundial de saúde

ORAC Oxygen radical absorbance capacity

ppm Partes por milhão

pH Potencial hidrogeniônico

PI Ponto isoelétrico

S Segundo

SDS-PAGE-Tricina - Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis- Tricina

SDS-PAGE 2D- Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis bidimensional

SDS Sodium lauryl sulfate

UFPB Universidade Federal da Paraíba

UNICAMP Universidade de Campinas

UV Ultravioleta

UNIFESP Universidade Federal de São Paulo

V voltagem

SUMÁRIO

1 INTRODUÇÃO

O inhame (*Dioscorea* spp.) pertencente a família Dioscoreaceae, apresenta cerca de 600 espécies, das quais, as de maior importância na alimentação humana são: *Dioscorea cayennensis, Dioscorea rotundata, Dioscorea alata, Dioscorea trífida e Dioscorea esculenta* (SANTOS, 2002). O tubérculo inhame encontra-se distribuido principalmente nos trópicos, sendo uma importante cultura na América do Sul, Ásia e África, constituindo-se como um alimento básico nestas regiões de cultivo (HAN et al., 2013).

Dentre as espécies, a cultivar inhame da costa (*D. cayennensis*) se destaca com maior consumo no Nordeste do Brasil (CARMO; 2002). Nesta região, este tubérculo representa alternativa viável para a agricultura, devido as condições ambientais favoráveis para a sua produção, além de se caracterizar como importante fonte de renda para os pequenos agricultores familiares, devido ao fácil manejo e robustez da cultura (LEONEL; CEREDA, 2002; OLIVEIRA et al, 2012).

Do ponto de vista nutricional, o inhame produz túberas de alto valor nutritivo e energético devido ao elevado teor de amido, contendo ainda vitaminas do complexo B (tiamina, riboflavina, niacina), minerais e apresenta baixos teores de gordura, além de apresentar propriedades medicinais milenar (FU; FERNG; HUANG, 2006; JU et al., 2014). Além das pesquisas direcionadas ao estudo dos carboidratos do inhame, as proteínas deste tubérculo também vêm se destacando em recentes estudos (MA et al., 2017; ZHANG et al., 2019).

Inicialmente, a dioscorina, principal proteína de reserva do gênero Dioscorea foi reportada pela primeira vez em *D. rotundata* (HARVEY; BOULTER, 1983). Posteriormente, Conlan et al. (1995), identificaram e isolaram as proteínas da espécie *D. cayennensis* (dioscorina A e B), que apresentam cerca de 85% do conteúdo total de proteínas solúveis deste tubérculo. Os tubérculos inhame apresentam teores proteicos que diferem de acordo com a espécie. A espécie *D. alata* apresenta teores de proteína que variam de 8,13% a 15,07% (HUANG et al., 2007); *D. batata* e *D. pseudojaponica* apresentam conteúdo expressivo de 11,6% a 16,6 b.s., respectivamente (FU; FERNG; HUANG, 2006). Olatoye; Arueya (2019) em estudo mais recente, verificaram teores entre 3,55% a 8,64% de proteína bruta em diferentes cultivares do *D. bulbifera.*

A partir da descoberta da dioscorina, o interesse no estudo desta proteína vem ganhando destaque no âmbito científico. Diversas pesquisas evidenciaram que a dioscorina isolada, as proteínas hidrolisadas, bem como, seus peptídeos sintéticos, exibem várias atividades biológicas *in vitro* e *in vivo*, incluindo imunomoduladora, antioxidante e anti-hipertensiva (LU et al., 2012; HAN et al., 2013; NAGAI et al., 2014; HAN et al., 2014). Porém, o conjunto das proteínas de inhame ainda não tiveram sua bioatividade explorada, após fragmentação por simulação da digestão gastrointestinal. Essa abordagem otimiza a liberação de peptídeos que podem ter sua atividade biológica potencializada, isso ocorre devido a exposição de grupos ionizáveis e resíduos de aminoácidos hidrofóbicos que têm ação em vários mecanismos biológicos (MÖLLER et al., 2008; ARCAN; YEMENICIOGLU, 2010). Neste sentido, a digestão gastrointestinal simulada *in vitro* pode contribuir para a liberação substancial de peptídeos biologicamente ativos.

Na últimas duas décadas, o efeito biológico de compostos naturais tem sido alvo de intensa investigação. Deste modo, um novo campo de pesquisa se abriu a partir de diversos estudos com fontes proteicas, para a obtenção de hidrolisados e peptídeos bioativos em alimentos. Estes peptídeos estão sendo foco de estudos, principalmente, pela variabilidade de aplicações biológicas que apresentam, dentre as quais destacam-se a anti-hipertensiva, antioxidante, anti-inflamatória e antimicrobiana (LI-CHAN, 2015). Diversas fontes de proteína alimentar de origem vegetal e animal têm se destacado como precursoras de peptídeos bioativos, tais como farelo de milho, ostras, soro de leite e ovos, pela potencialidade de seus efeitos biológicos (CARVALHO-SILVA et al., 2012; ZHUANG; TANG; YUAN, 2013; WANG et al., 2014; MAJUMDER et al., 2015).

Diante das evidências, é notória a importância da busca e obtenção de novos peptídeos, pela gama de aplicações biológicas e futuro promissor como compostos bioativos naturais, que podem contribuir na saúde humana e no desenvolvimento de novos ingredientes funcionais. Neste sentido, o estudo da funcionalidade biológica das proteínas do inhame (*D. cayennensis*) se apresenta com caráter inovador de pesquisa científica. Portanto, o presente estudo objetivou caracterizar e produzir o concentrado proteico, bem como realizar a prospecção de peptídeos bioativos obtidos através da digestão gastrointestinal simulada *in vitro* das proteínas do inhame (*D. cayennensis)*, cultivado no Nordeste do Brasil.

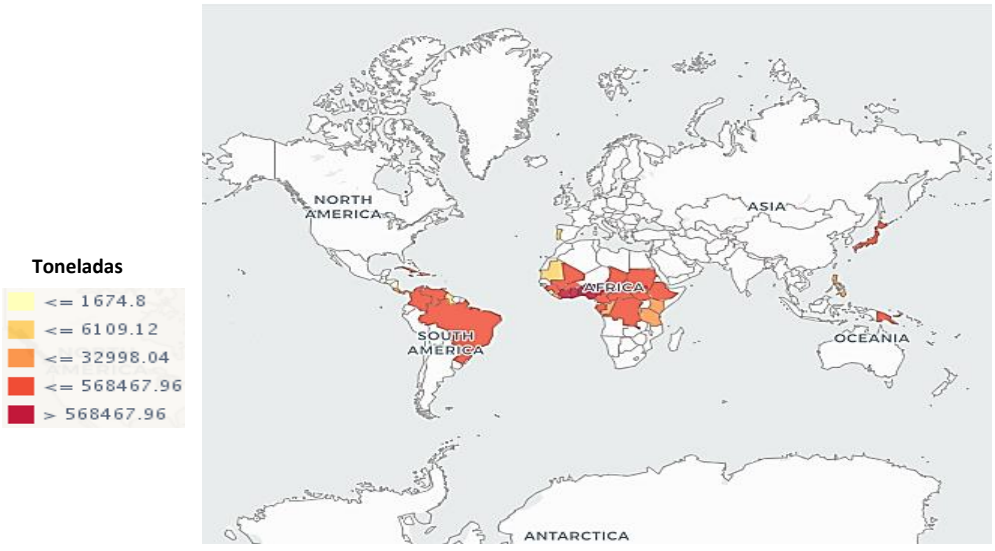
2 REVISÃO DA LITERATURA

2.1 TUBÉRCULO INHAME

As culturas tuberosas, que em sua maioria apresentam o amido como componente principal de armazenamento, formam um grupo bem diversificado de plantas produtoras de bulbos, raízes e tubérculos. A distribuição dessas plantas ocorre principalmente nas regiões tropicais, em que o cultivo é favorecido pelas condições ambientais, além da valorização como meio de subsistência e da importância econômica, étnica e cultural (PEDRALLI, 2002). Dentro do grupo das tuberosas, o cultivo de inhame (*Dioscorea* spp.) se destaca no Brasil, com os principais campos de produção localizados principalmente na região Nordeste do país (SANTOS; MACEDO, 2002).

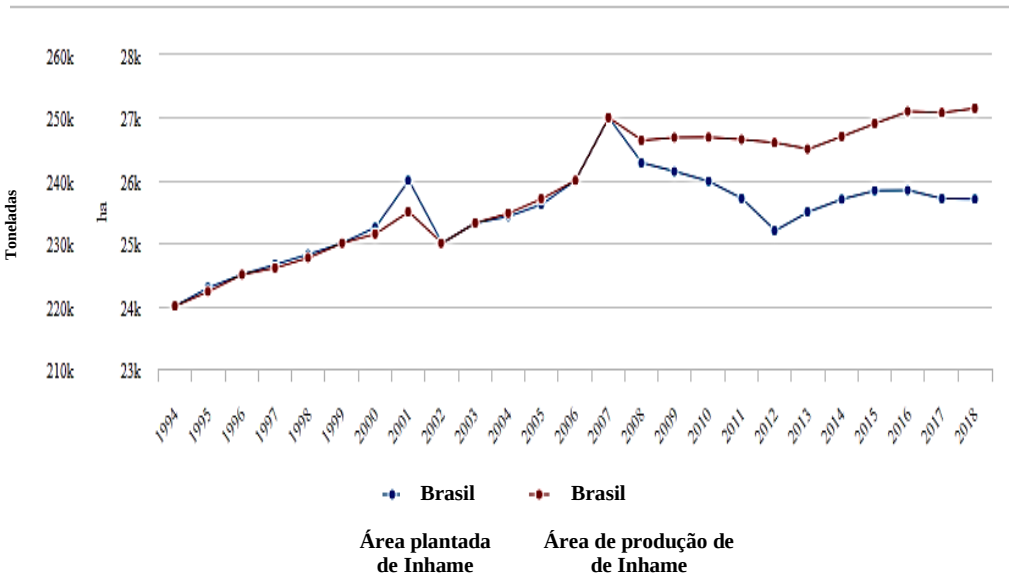
O cenário mundial de produção do inhame encontra-se apresentado na Figura 1, onde constata-se que o continente africano se apresenta como maior produtor, com percentual de 96,4%, com destaque para a Nigéria, com mais de 44 milhões de toneladas em 2018. Dentre os países da América do Sul, o Brasil ocupa o primeiro lugar na produção deste tubérculo, com estimativa em 2018 de aproximadamente 251 mil toneladas distribuídas em uma área de 25 mil hectares, como pode ser verificado na Figura 2 (FAOSTAT, 2020).

Figura 1. Mapeamento da produção mundial de inhame (1994-2018)



Fonte: FAOSTAT (2020)

Figura 2. Produção/Rendimento de inhame no Brasil (Média de 1994-2018)



Fonte: FAOSTAT (2020)

No panorama brasileiro, de acordo com os dados da FAOSTAT (2020), ocorreu uma evolução ascendente na produção do inhame ao longo dos anos de 1994 até 2018 (Figura 2). A cultura do inhame apresenta grande importância no hábito de consumo alimentar e aspecto socioeconômico no Brasil, principalmente na região nordeste, sobretudo, para os estados de Sergipe, Paraíba, Pernambuco, Alagoas, Bahia e Maranhão (MOURA, 2002; MENDES, 2005), tendo como espécies predominantes: *D. cayennensis e D. alata*. Os estados de Pernambuco e Paraíba se apresentam como os maiores produtores de inhame a nível nacional (OLIVEIRA et al., 2012). O consumo deste tubérculo é bastante apreciado na culinária nordestina, seja na forma cozida ou em preparações de sopas e purês, como também, farinha para misturas na produção de pães (MONTEIRO, PERESSIN, 2002).

O inhame apresenta relevância como um alimento de qualidade nutritiva e energética, assim como alimento de qualidade funcional. Isso devido a presença de pigmentos, conforme refere Muimba-Kankolongo (2018), que o *D. cayennensis* é popularmente conhecido na África como "inhame amarelo", devido a presença de carotenoides nestes tubérculos (MUIMBA-KANKOLONGO, 2018). Assim como em outras raízes tuberosas, o inhame é fonte de amido, com um considerável teor deste carboidrato (18-25%) (BHANDARI; KASAI; KAWABATA, 2003). Além disso, se destaca como fonte de fibra solúvel

(mucilagem) e baixo teor de lipídeos. Apresenta ainda, vitaminas do complexo B (tiamina, riboflavina, niacina), ferro e compostos fenólicos (AMANI et al., 2004; KAWABATA, 2004; BHANDARI; HUANG et al., 2007; SANTOS et al., 2007). Apresenta cerca de 1 a 3% de proteínas em seus tubérculos frescos. Em base seca, este teor de proteína bruta aumenta para 6 a13% (LU et al., 2012).

2.2 BIOATIVIDADE DAS PROTEÍNAS DO TUBÉRCULO INHAME

Harvey e Boulter (1983), foram os pioneiros na investigação das proteínas de Dioscorea spp. Estes pesquisadores isolaram as proteínas de reserva do inhame (dioscorina A e B), cujo conteúdo apresenta cerca de 85% de proteínas solúveis e representam as principais proteínas deste tubérculo. Fu; Ferng; Huang (2006), ao estudarem as espécies *Dioscorea pseudojaponica* e *Dioscorea batatas*, verificaram que as mesmas apresentam um bom conteúdo de proteínas (16,6 e 11,6%) b.s., respectivamente. Ao longo dos anos a proteína dioscorina tem sido extensivamente estudada e caracterizada. Recentemente, Lin et al. (2017), isolaram cinco genes de dioscorina de *D. alata* L., compreendendo três classes A (Da-dio1,-3 e -4) e duas isoformas de classe B (Da-dio2 e -5). Além da caracterização, as pesquisas têm evidenciado a funcionalidade das proteínas de diferentes espécies de Dioscorea spp. As atividades biológicas da proteína dioscorina encontram-se sumarizadas no Quadro1.

Quadro 1. Atividades biológicas da proteína dioscorina de diferentes espécies de *Dioscorea* spp.

Atividade biológica	Espécie	Referência
Anti-hipertensiva	*D. alata cv.* Tainong No. 1 *D.oposita*	HSU et al., 2002; LIN et al., 2006; LIN et al., 2014 NAGAI et al., 2006
Antioxidante	*D. alata* L. cv. Tainong *D. cayennensis* *D. alata L. var. purpurea* *D. alata L. var. Tainung* *D. thunb. var. pseudojaponica* *D.oposita* *D. batatas* Decner	HAN et al., 2014 HAN et al., 2013 LIU et al, 2009 LIU et al, 2009 LIU et al, 2009 NAGAI et al., 2006 HOU et al. 2001
Imunoduladora	*D. thunb. var. pseudojaponica* *D. alata cv.* Tainong No. 1 *D. alata* *D. japonica*	LIU et al, 2007 LIU et al, 2007 FU et al., 2006; LIN et al., 2009 LIN et al., 2009
Anti-inflamatória	*D. batatas* Decner *D. alata; D. japônica* *D. nipponica Makinoand*	HSU et al., 2013; YANG; LIN, 2014 HSU; HUN; LIN, 2015 WU et al., 2015
Antialérgica	*D. japonica* *D. cayennensis*	JHENG et al., 2012 FU et al., 2009

Dentre as atividades biológicas apresentadas no Quadro 1, encontra-se a anti-hipertensiva *in vitro* das dioscorinas A e B, por inibição da enzima conversora de angiotensina (HSU et al., 2002). Além disso, a confirmação do efeito anti-hipertensivo foi constatado *in vivo,* nos estudos realizados por Lin et al. (2006) e Lin et al., (2014), com a administração oral de dioscorina purificada, bem como, dos hidrolisados proteicos e peptídeos sintéticos, apresentando efetiva redução da pressão arterial de ratos espontaneamente hipertensos.

Os pesquisadores Hou et al. (2001) relataram que a dioscorina exibiu atividade sequestradora *in vitro* contra radicais DPPH e hidroxila, sendo este efeito dose-dependente. No mesmo estudo, verificou-se ainda que a atividade antioxidante foi favorecida pela presença de cisteína na estrutura dos peptídeos. No estudo desenvolvido por Liu e Lin (2009), as dioscorinas foram hidrolisadas pela enzima pepsina, utilizada para simular a digestão gástrica *in vivo,* e como resultado, verificou-se que a atividade de eliminação do radical DPPH, aumentava com hidrolisados com maior tempo de hidrólise e

consequentemente, com menor peso molecular. Outros estudos de simulação computadorizada da hidrólise da dioscorina com produção de peptídeos sintéticos, corroboram para o fato de que esta molécula quando fragmentada apresentou capacidade antioxidante, com potencial como composto bioativo (HAN et al., 2013; HAN et al., 2014).

Além destas funções, pesquisas também mostram que a dioscorina de diferentes espécies de Dioscorea spp, tem efeito imunomodulatório por meio de diversos mecanismos, dentre os quais a modulação da sinalização e indução da expressão de citocinas pró-inflamatórias. Esta proteína também tem participação em importantes vias de sinalização para a atividade imunológica contra micro-organismos e aumento da fagocitose de bactérias patogênicas (LIU et al., 2007; LIN et al., 2009). Os pesquisadores Wu et al. (2015) verificaram que esta proteína foi responsável pela inibição da inflamação, por meio da interrupção de vias de sinalização que promovem a resposta inflamatória. Deste modo, esta proteína tem potencial para contribuir no tratamento de doenças inflamatórias, atuando como composto anti-inflamatório.

O estudo de Zhang et. al (2019) referem que as proteínas e peptídeos do inhame possuem potencial efeito terapêutico no tratamento de doenças cardiovasculares, inflamatórias, diferentes tipos de cânceres, distúrbios do envelhecimento, menopausa e osteoporose. Nesse sentido, a pesquisa *in vivo* realizada por Lok Wong et al. (2015) verificou que a proteína do *D. opposita* apresentou atividade estimuladora do estrogênio, com diminuição dos sintomas da síndrome da menopausa. Esta pesquisa também identificou a atividade osteogênica, com progressão neutralizadora da osteoporose e consequente aumento da densidade mineral óssea. Ademais, conforme Lu et al. (2012) a dioscorina e seus peptídeos são recursos potenciais para o desenvolvimento de alimentos funcionais, diante das diversas atividades biológicas apresentadas, e ainda, são alvos promissores para pesquisadores de proteínas alimentares.

Com base nos efeitos destas moléculas, este tubérculo tem sido considerado um alimento funcional, podendo ser aproveitado como ingrediente para o desenvolvimento de produtos alimentícios para a promoção da saúde. Desta forma, outras investigações são necessárias com o estudo do conjunto das proteínas do inhame, suas estruturas, fragmentação proteica por hidrólise, descobertas sobre novas atividades biológicas, assim como seus mecanismos de ação.

2.3 DIGESTÃO GASTROINTESTINAL SIMULADA DE PROTEÍNAS *IN VITRO*

O processo de digestão de alimentos é considerado complexo, pois envolve muitos fatores diretamente relacionados com o aproveitamento dos alimentos pelo organismo humano e os benefícios que estes podem promover para a saúde (BORNHORST et al., 2016). Porém, devido à complexidade inerente ao processo *in vivo,* para a elucidação da biodisponibilidade dos alimentos, os estudos têm sido direcionados para a investigação da digestão simulada *in vitro,* buscando esclarecer os benefícios provenientes dos alimentos. Nesse sentido, os ensaios de digestão *in vitro* são utilizados para simular as condições fisiológicas da digestão *in vivo,* sendo considerada uma ferramenta útil para estudar e compreender as mudanças, interações, bem como a bioacessibilidade de nutrientes, drogas e compostos não nutritivos (EGGER; MÉNARD, 2017).

Diversas pesquisas com a utilização da digestão gastrointestinal (GI) simulada *in vitro* têm sido publicadas. Estes estudos visam elucidar muitos aspectos, tais como a digestibilidade proteica, interações entre nutrientes, dentre outros. Esta técnica tem sido amplamente utilizada em áreas como nutrição, farmacologia e química alimentar (LUCAS-GONZALES et al., 2018). Sanchón et al. (2018) seguindo protocolo estabelecido pela INFOGEST (2014), realizaram um comparativo entre digestão gastrointestinal *in vitro* e *in vivo* e como resultado deste estudo, verificaram que a digestão *in vitro* constitui-se com boa aproximação à digestão gastrointestinal fisiológica *in vivo*. A tendência de utilização da digestão de proteínas por simulação GI *in vitro,* envolve a liberação de peptídeos, bem como a investigação destes como compostos biologicamente ativos. Nesse sentido, as pesquisas têm mostrado que peptídeos bioativos podem ser gerados de diferentes níveis de degradação proteica, incluindo a digestão *in vitro* (KOPF-BOLANZ, 2014) ou *in vivo* (BOUTROU, HENRY, SANCHEZ-RIVERA, 2015).

Em estudo recente realizado com produtos derivados do leite de búfala, Basilicata et al. (2018) submeteram estes produtos à digestão gastrintestinal simulada *in vitro*. Após a digestão e liberação de fragmentos peptídicos, todos os peptídeos resultantes foram caracterizados por espectrometria de massas e submetidos à pesquisa de bioatividade. Como resposta, foram identificados grande número de potenciais peptídeos bioativos com várias atividades biológicas, tais como anti-hipertensivos, imunomoduladores e antioxidantes.

As proteínas de origem vegetal quando fragmentadas por digestão, também se destacam como promissoras nos benefícios à saúde. O estudo de Vilcacundo et al. (2018) comprovou o potencial das proteínas de quinoa como excelentes precursoras de peptídeos bioativos. A digestão gastrointestinal *in vitro* destas proteínas resultou na liberação de uma variedade de peptídeos que podem ser considerados bioativos, devido a constatação das atividades sequestrantes de radicais livres (fração < 5 kDa) e anticâncer (fração >5 kDa). Em outro estudo, realizado por González-Montoya et al. (2018), foi verificado que os peptídeos provenientes da proteína de soja fragmentada por digestão GI simulada *in vitro*, exibiram excelente atividade antidiabetes.

Diante desse campo de pesquisa, a simulação da digestão GI *in vitro*, aponta para a influência positiva que estes alimentos podem proporcionar após sua ingestão e digestão no organismo humano. A composição diferencial e a abundância de potenciais peptídeos liberados *in vitro*, fornecem um reservatório de sequências bioativas semelhantes as liberadas *in vivo*, com potencial na prevenção de doenças degenerativas e consequente benefícios à saúde.

2.4 PEPTÍDEOS BIOATIVOS EM ALIMENTOS

Os peptídeos são definidos como fragmentos de proteínas específicas que podem ter impacto positivo nas funções ou condições do organismo humano, podendo assim, ter influência na saúde (KITTS e WEILER, 2003). Estes peptídeos estão inativos dentro da matriz proteica, sendo liberados por diferentes processos de hidrólise, os quais podem torná-los fisiologicamente ativos (Capriotti et al., 2016). A hidrólise inclui, digestão *in vivo*, que ocorre no trato gastrointestinal por enzimas humanas e microbiota; processamento de alimentos *in vitro*, e ainda por maturação de alimentos por culturas de micro-organismos (KORHONEN, 2009).

Os peptídeos bioativos de alimentos se caracterizam por apresentarem sequências de aminoácidos, que geralmente, contém de 3 a 20 aminoácidos (MANIKKAM et al., 2016). Estes têm exibido diferentes atividades biológicas que estão relacionadas com sua estrutura, composição e sequência de seus aminoácidos. Devido seus efeitos biológicos, os peptídeos bioativos têm atraído cada vez mais à atenção, por contribuir na prevenção de doenças degenerativas e consequente promoção da saúde humana (UDENIGWE; ALUKO, 2012; TU

et al., 2018). Diversos efeitos biológicos têm sido atribuídos aos peptídeos oriundos de proteínas de origem alimentar. As principais atividades biológicas estão descritas no Quadro 2.

Quadro 2. Atividades biológicas de peptídeos e hidrolisados de diferentes fontes proteicas de alimentos de origem vegetal

Atividade biológica	Fonte proteica	Modelo experimental	Sequência peptídica/hidrolisado	Referência
Antioxidante	Batata doce	*in vitro*	HDSASGQY YYMVSA HDSESGQY YYIVS RYYDPL	ZHANG; MU, 2017
	Batata doce	*in vitro*	Frações peptídicas	ZHANG; MU, 2016
	Batata doce	*in vitro*	TYQTF, SGQTFL, YMVSAIWG, YYIVS, YYDPL	ZHANG; MU; SUN, 2014
	Sementes de chia	*in vitro*	Hidrolisado proteico e frações peptídicas	SEGURA-CAMPOS et al., 2013; COELHO et al., 2019
	Feijão Bambara	*in vitro*	Hidrolisado proteico	MUNE; MINKA; HENLE, 2018
	Amaranto	*in vitro*	YLAGKPQQEH	DELGADO et al., 2015
	Amaranto	*in vitro*	IYIEQGNGITGM TEVWDSN	VILCACUNDO et al., 2018
	Amaranto	*in vitro*	REQGSR	DELGADO et al., 2016
	Trigo Tremoço Ervilha	*in vitro*	VLPQQQY TVTSLDLPVLRW VTSLDLPVLRW, FVPY	BABINI et al., 2017
	Quinoa	*in vitro*	FLISCLL, SVFDEELS, DFIILE	VILCACUNDO et al., 2017
	Glúten	*in vivo*	Frações de peptídeos	LIU et al., 2015
	Trigo	*in vitro*	Hidrolisado proteico	ZHU et al., 2006
	Arroz	*in vitro*	VHHH, Frações pépticas	PHONGTHAI et al., 2018
	Cacau	*in vivo*	DNYDNSAGKWWVT	MARTORELL et al., 2013

Anti-hipertensiva	Glúten de trigo	*in vitro*	SAGGYIW, APATPSFW	ZHANG et al., 2020
	Amaranto	*in vitro/in vivo*	Hidrolisado proteico	FRITZ et al., 2011
	Lentilha	*in vitro*	Hidrolisado proteico	BARBANA et al., 2011
	Arroz	*in vitro*	VWP, VNP	CHEN et al., 2013
	Arroz	*in vivo*	Hidrolisado proteico	CHEN et al., 2007
	Farelo de arroz	*in vitro*	YSK	WANG et al., 2017
	Canola	*in vitro*	FL, VSV	WU; ALUKO; MUIR, 2008
	Soja Feijão	*in vitro, in vivo* *in vivo*	YLAGNQ, FFL, IYLL KDYRL, VTPALR, KLPAGTLF	CHEN et al., 1998 LI et al., 2006
	Ervilha	*in vitro*	IR, KF, EF, LR, NR, FT	LI; ALUKO, 2010
	Sementes de chia	*in vitro*	Hidrolisado proteico	SEGURA-CAMPOS et., 2013
	Amaranto	*in vitro*	FLISCLL, SVFDEELS, DFIILE	VILCACUNDO et al., 2018
	Sementes de Nozes	*in vitro*	WPERPPQIP	LIU et al., 2013
	Girassol	*in vitro*	FVNPQAGS	MEGÍAS et al., 2004
	Sementes de palma	*in vitro*	VVLYK	ZHENG et al., 2017
	Batata	*in vitro, in vivo*	Frações peptídicas	MÄKINEN et al., 2016
	Batata doce	*in vitro*	ITP, IIP, GQY, STYQT	ISHIGURO et al., 2012

Hipoglicemiante	Quinoa	*in vitro*	Hidrolisado proteico	NONGONIERMA et a., 2015
	Arroz	*in vitro*	Hidrolisado proteico	HATANAKA et al., 2012
	Quinoa	*in vitro*	IQAEGGLT, DKDYPK, GEHGSDGNV	VILCACUNDO et al., 2017
	Milho	*in vivo*	Hidrolisado proteico	MOCHIDA; HIRA; HARA, 2010
	Feijão preto	*in vitro*	TTGGKGGK	MOJICA; MEJÍCA, 2016
	Arroz, Ervilha, soja	*in vitro*	Hidrolisado proteico	NONGONIERMA; FITZGERALD, 2015
	Sementes de cominho	*in vitro*	FFRSKLLSDGAAAAKGALLPQYW, RCMAFLLSDGAAAAQQLLPQYW	SIOW; GAN, 2016
Imunomoduladora	Soja	*in vivo*	Hidrolisado proteico	EGUSA; OTANI, 2009
	Soja	*in vitro*	VPY	KOVACS-NOLAN et al., 2012
	Amaranto	*in vivo*	SSEDIKE	MORONTA et al., 2016
	Semente de linhaça	*in vitro*	Hidrolisado proteico	SILVA et al., 2018
	Glúten de trigo	*in vitro*	Hidrolisado proteico	WENJIA et al., 2016
Antimicrobiana	Soja	*in vitro*	PGTAVFK	MCCLEAN; BEGGS; WELCH, 2014
	Feijão	*in vitro*	KTCENLADTY~	WONG; NG, 2005
	Sementes de goiaba	*in silico*	MILI e outros	PORTO et al., 2018
	Sementes de chia	*in vitro*	Hidrolisado proteico	SEGURA-CAMPOS et al., 2013

De acordo com Martinéz-Augustin et al. (2014) e Li-Chan (2015), os peptídeos bioativos atuam na prevenção do estresse oxidativo, redução da pressão arterial, prevenção de diabetes tipo 2, como anti-inflamatório, assim como em várias doenças crônicas degenerativas. Com base no Quadro 2, as principais atividades biológicas dos peptídeos estão descritas nos tópicos a seguir:

2.4.1 Atividade antioxidante

O processo de estresse oxidativo é causado pelo acúmulo de radicais no organismo humano. Este processo é ocasionado devido ao desequilíbrio entre a produção e eliminação de espécies reativas de oxigênio, nitrogênio e enxofre (LI et al., 2015). Neste sentido, isso implica em vários distúrbios no organismo humano, que podem levar às doenças crônicas degenerativas (MATSUDA; SHIMOMURA, 2013). Porém, os antioxidantes oriundos da alimentação auxiliam no combate aos radicais livres, com redução do estresse oxidativo, contribuindo para promoção da saúde. Portanto, a atividade antioxidante é um dos atributos de promoção da saúde, atribuída inclusive aos peptídeos antioxidantes, obtidos a partir de proteínas alimentares (SARMADI; ISMAIL, 2010; LI-CHAN, 2015).

Dentre os estudos com hidrolisados e peptídeos que apresentam atividade antioxidante, encontram-se as pesquisas com proteínas vegetais, tais como sementes de quinoa (VILCACUNDO et al., 2017), batata doce (ZHANG; MU, 2017) sementes de chia (COELHO et al., 2019; SEGURA-CAMPOS et al., 2013), linhaça (SILVA et al., 2012) dentre várias outras resumidas no Quadro 2.

A capacidade antioxidante dos peptídeos está diretamente relacionada à presença de alguns aminoácidos em sua estrutura, sequência destes, bem como peso molecular e conformação (SAMARANAYAKA; LI-CHAN, 2011). Estas características possibilitam os mecanismos de ação dos peptídeos, como postulado por Chen et al., (1998) com peptídeos da proteína de soja, em que a posição do grupo imidazol no aminoácido histidina possibilita a doação de protóns de hidrogênio para estabilizar moléculas oxidantes. Os aminoácidos aromáticos (tirosina, fenilalanina e triptofano) assim como, o grupo SH de cisteína, também podem doar prótons aos radicais livres e quelar íons metálicos pró-oxidantes, com consequente estabilização destas moléculas (SARMADI;

ISMAIL, 2010; UDENIGWE; ALUKO, 2012). Babini et al. (2017) constataram que a presença de um resíduo de triptofano no C-terminal dos peptídeos TVTSLDLPVLR**W**, VTSLDLPVLR**W** e TSLDLPVLR**W** conferiu maior atividade antioxidante, a partir da atividade sequestrante do radical ABTS.

De acorco com Nwachukwu e Aluko (2019) a capacidade antioxidante dos peptídeos é determinada a partir de ensaios *in vitro*, com base na capacidade de sequestrar radicais livres (2,2 - difenil - 1-picrylhydrazyl, hydroxyl, superóxido), pelo poder redutor do ferro férrico a ferroso, pela ligação à metais e inibição da oxidação lipídica. Com base nestes estudos, Morisco et al. (2004) relatam que hidrolisados de proteínas vegetais, podem ter eficácia na prevenção e tratamento de várias doenças relacionadas ao estresse oxidativo. Neste sentido, os peptídeos possuem potencial para serem utilizados como antioxidantes naturais, a fim de retardar processos oxidativos no organismo humano, bem como em alimentos (ELIAS, 2008; SARMADI; ISMAIL, 2010).

Estudos também identificaram a capacidade antioxidante dos peptídeos através de ensaios com culturas de células, assim como em modelo animal. Nesse aspecto, Liu; Finley (2005) verificaram que peptídeos antioxidantes são capazes de retardar a oxidação celular *in vivo,* podendo contribuir para redução do risco do desenvolvimento de doenças crônicas não transmissíveis. Visando o avanço das pesquisas, o estudo de Kong et al. (2012) constatou que o peptídeo VHLKP, obtido da proteína do leite, foi capaz de proteger linhagens de células MRC-5 contra radicais livres, com redução significativa de danos causados por H_2O_2 e morte celular. Ademais, os estudos com culturas de células constituem um passo necessário para o avanço de pesquisas com animais, assim como ensaios clínicos em humanos. Para tanto, Mada et al. (2017) verificaram a redução de danos oxidativos em células de ratos a partir de experimentos com o peptídeo VLPVPQK, obtido do leite de búfala. De acordo com Devi et al. (2019) os aminoácidos hidrofóbicos e a carga positiva deste peptídeo possibilitaram o mecanismo de ação contra os radicais livres. Esses achados estabelecem evidências do potencial dos peptídeos de proteína alimentar, que podem atuar como composto antioxidante natural, atenuando o processo de estresse oxidativo.

2.4.2 Atividade anti-hipertensiva

A hipertensão é uma desordem caracterizada por relaxamento insuficiente dos vasos sanguíneos e fluxo sanguíneo reduzido por estreitamento de vasos e arterias. Esta patologia se caracteriza como sendo um problema de saúde pública e está associada à várias outras doenças crônicas tais como obesidade, diabetes, aterosclerose, dentre outras (LI et al., 2014). De acordo com Acharya et al., 2003, a pressão arterial é regulada pelo sistema renina-angiotensina. Essa via regula a contração dos vasos sanguíneos, em que a renina converte o angiotensinogênio em angiotensina I (Ang I), pela ação da enzima conversora de angiotensina (ECA), ao passo que a Ang I é convertida em angiotensina II (Ang II) que se liga aos receptores na parede vascular para causar contração dos vasos sanguíneos. Em condições anormais, ocorre aumento da atividade da renina ou da ECA, que provoca níveis elevados de Ang II e ocasiona a contração excessiva dos vasos sanguíneos, e consequente desequilíbrio entre contração e relaxamento (ALUKO, 2015).

Com base nestes fatores que são responsáveis pela elevação da pressão arterial, os tratamentos medicamentosos tradicionais utilizam princípios ativos para a inibição das atividades da renina e/ou ECA, como Capoten e Casotec, dentre outros que são comercializados. Porém, vale ressaltar que a maioria destes fármacos estão regularmente associados à reações adversas (ABASSI; WINAVER; FEUERSTEIN, 2009). Sendo assim, as pesquisas na área da saúde têm sido direcionadas para a descoberta de compostos alimentares naturais com atividade inibidora da ECA ou renina, que sejam seguros e reduzam os efeitos colaterais que estão associados aos fármacos tradicionais (ALUKO, 2015).

A atividade anti-hipertensiva dos peptídeos tem sido extensivamente estudada e as pesquisas têm demostrado que estes fragmentos proteicos podem modular o controle da pressão sanguínea, a partir da inibição da ECA (VERMEIRSSEN; CAMP; VERSTRAET, 2004). De acordo com Fujita; Yoshikawa (1999), o mecanismo de ação dos peptídeos anti-hipertensivos é influenciado pela presença dos aminoácidos prolina, leucina e isoleucina. Danish et al. (2017) comprovaram a eficiência de peptídeos inibidores da ECA contendo prolina e leucina. Enquanto Zhang et al. (2020) verificaram que os aminoácidos aromáticos também podem potencializar este efeito.

Dentre os estudos resumidos no Quadro 2, destaca-se a pesquisa *in vitro* realizada por Zhang et al. (2020), na qual foram identificados novos peptídeos inibidores da ECA, obtidos da hidrólise das proteínas do glúten de trigo. Assim como o estudo de Vilcacundo et al. (2018), com a obtenção de peptídeos anti-hipertensivos oriundos da proteína de amaranto. Além do estudo em modelo experimental *in vivo*, com a administração de frações peptídicas da batata, em camundongos espontaneamente hipertensos que resultou na inibição da ECA (MÄKINEN et al., 2016). Diante deste contexto, os peptídeos inibidores da ECA podem ser uma alternativa viável para auxiliar no tratamento da hipertensão.

2.4.3 Atividade hipoglicemiante

O diabetes mellitus (DM) consiste em um distúrbio metabólico crônico, cracterizado por hiperglicemia persistente, que ocorre com a disfunção do pâncreas na produção de insulina ou quando o corpo não usa efetivamente a insulina que produz. Ademais, é considerado um dos principais problemas de saúde global, devido o crescimento de sua incidência e prevalência (OMS, 2018). Os fatores genéticos, biológicos e ambientais são considerados os principais causadores dos diferentes tipos de DM (tipo 1, 2 e gestacional) (COURI; FOSS; VOLTARELLI, 2006). O DM tipo 2 possui etiologia complexa e multifatorial, com perda progressiva de secreção insulínica combinada com resistência à insulina (AMERICAN DIABETES ASSOCIATION, 2017).

No organismo humano, no metabolismo dos açúcares, os hormônios incretinas são liberados em resposta à presença de nutrientes no lúmen intestinal e atuam estimulando a secreção de insulina nas células pancreáticas, a fim de manter a regulação da glicose sanguínea (HOLST; DEACON, 2004). Porém, em indivíduos diabéticos a enzima dipeptidil peptidase IV (DPP-IV), causa rápida degradação e inativação dos hormônios insulonotrópicos, com implicação nos níveis normais da glicose na corrente sanguínea (MENTLEIN, 2005). Portanto, a inativação da DPP-IV se configura como uma das abordagens para regular os níveis de glicose no sangue.

Nessa perspectiva, os peptídeos bioativos têm sido reportados como reguladores da secreção de DPP-IV, exercendo influência benéfica sobre os níveis de glicemia

(CICERO, FOGACCI, COLLETI, 2016). Conforme apresentado no Quadro 2, estudos *in vitro* referem que os peptídeos possuem efeito na inibição do DPP-IV. São exemplos os peptídeos de quinoa (VILCACUNDO et al., 2017) e feijão (MOJICA; MEJÍCA, 2016). Assim como também em estudo *in vivo*, com a administração do hidrolisado de milho e atenuação da glicemia de ratos (MOCHIDA; HIRA; HARA, 2010). Portanto, peptídeos e hidrolisados de proteínas vegetais também são compostos promissores como agentes hipoglicemiantes e podem contribuir para auxiliar no controle do DM tipo 2.

2.4.4 Atividade antibacteriana

Com o surgimento da resistência bacteriana a determinados antibióticos, constatou-se a necessidade de pesquisas e desenvolvimento de novos compostos antimicrobianos (WILLIAMS; BAX, 2009). Dentre os compostos estudados como alternativas antimicrobianas encontram-se os peptídeos. Gobbetti et al. (2007) afirma que os peptídeos antimicrobianos são liberados após hidrólise enzimática, especialmente durante a digestão gastrointestinal. De acordo com Nguyen; Haney; Vogel (2011), estes peptídeos têm sido reconhecidos como alternativas promissoras com amplo espectro de atividade contra agentes patogênicos, como bactérias e fungos.

Deste modo, peptídeos de origem vegetal e animal, têm sido reconhecidos por sua atividade antibacteriana. Conforme Meisel (1998) e Mohanty et al. (2016) os peptídeos de leite e derivados lácteos apresentam boas fontes de peptídeos antibacterianos, que são oriundos das proteínas do soro e caseína, assim como os peptídeos bacteriocinas (TAHIRI et al., 2009). No estudo realizado por Demers-Mathieu et al. (2013), foi verificada a inibição do crescimento de bactérias patogênicas (*L. monocytogenes* e *S. aureus*) a partir do uso da fração proteica do leite, obtida após hidrólise com a enzima tripsina. Outros peptídeos antibacterianos obtidos de proteína vegetal também são reportados na literatura, tais como o peptídeo lunatusin, oriundo das proteínas do feijão lima (WONG; NG, 2005); peptídeos das sementes de trigo (FUJIMURA et al., 2003) e da proteína de milho (DUVICK et al., 1992) dentre outros.

Para desempenho de seu mecanismo de ação, os peptídeos antibacterianos necessitam de algumas propriedades essenciais, tais como carga catiônica, o que promove seletividade para as membranas bacterianas com cargas negativas. Além disso, uma

proporção significativa de resíduos de aminoácidos hidrofóbicos facilita as interações com as cadeias de ácidos graxos (BLONDELLE; LOHNER; AGUILAR, 1999). Desta forma, os peptídeos antibacterianos podem interagir com as membranas das células bacterianas e formar poros transmembranares que causam extravasamento descontrolado de íons e outros conteúdos da célula, levando assim à morte celular e consequente morte da bactéria (MANI et al., 2006). Os peptídeos antibacterianos também atuam na inibição de importantes vias dentro da célula, como replicação do DNA e síntese de proteínas (BROGDEN, 2005; LE et al., 2017).

De acordo com Wang; Yue; Lee (2015), os peptídeos antibacterianos podem ser facilmente digeridos no intestino. Assim, a proteína oriunda de alimentos, digerida libera peptídeos antibacterianos, que podem ser utilizados pelo organismo humano. Esse progresso para a descoberta de compostos antibacterianos de origem alimentar com propriedades biológicas, oferece excelentes oportunidades para a expansão do uso de alimentos no tratamento ou prevenção de doenças, sobretudo com os peptídeos bioativos que podem contribuir de forma positiva para a saúde humana.

2.5 BASES DE DADOS PARA PEPTÍDEOS BIOATIVOS EM ALIMENTOS

Os peptídeos bioativos derivados de alimentos têm sido reconhecidos como promissores como tratamentos alternativos, devido os resultados de suas atividades terapêuticas. Porém, os métodos convencionais para o isolamento e descoberta da bioatividade dos peptídeos, geralmente demandam tempo e alto custo (AGYEI; BAMBARANDAGE; UDENIGWE, 2019). Portanto, outras estratégias vêm sendo utilizadas para a obtenção e identificação de peptídeos bioativos. Nessa perspectiva, a abordagem *in silico* tem sido uma alternativa, visando a redução de custos e tempo no isolamento de peptídeos bioativos. Ademais, tem sido utilizada como método de predição do potencial bioativo de peptídeos oriundos de fontes alimentares, com contribuição significativa no processo de descoberta e identificação destes compostos bioativos (NONGONIERMA et al., 2014).

A abordagem *in silico* envolve inicialmente a fragmentação de proteínas conhecidas e descritas em bancos de dados, que incluem UniProt Consortium, National Center for Biotechnology Information e Research Collaboratory for Structural

Bioinformatics, a fim de identificar o potencial destas proteínas como fontes de peptídeos (AGYEI; BAMBARANDAGE; UDENIGWE, 2019). Nesse aspecto, várias bases de dados têm sido utilizadas para obtenção e pesquisa de peptídeos, resultando em uma coleção de peptídeos com ampla variedade de bioatividades (IWANIAK et al., 2019; UDENIGWE, 2014).

Dentre as bases de dados, a BIOPEP é a única que concentra principalmente peptídeos de origem alimentar, contendo registros de mais de 1500 peptídeos bioativos. A BIOPEP foi desenvolvida na Universidade de Warmia e Mazury na Polônia, esta ferramenta oferece ao usuário informações acerca de sequências de peptídeos com atividades biológicas, tais como antioxidante, antimicrobiana, anticâncer, anti-hipertensiva, antidiabetes, dentre outras (MINKIEWICZ et al. 2008). Além destas informações obtidas nesta base de dados, o *Peptide Ranker* permite inferir a probabilidade de um peptídeo apresentar bioatividade. Através desta ferramenta é atribuida uma pontuação para cada peptídeo, dentro do intervalo 0 a 1; onde quanto mais próximo de 1, maior é a probabilidade do peptídeo ser bioativo. Para prever diferentes classes de peptídeos bioativos, o *Peptide Ranker* considera características tais como o impacto do *status* extracelular e a composição de aminoácidos (MOONEY et al., 2012).

Adicionalmente, estas ferramentas permitem a predição dos mecanismos de ação dos peptídeos, bem como a influência da função biológica dependente de sua estrutura (KESKA; STADNIK, 2017; NONGONIERMA et al., 2014). Para tanto, são utilizados métodos computacionais e ferramentas estatísticas para a coleta e análise de dados bioquímicos, bem como modelos de design para predição do comportamento dos sistemas bioquímicos (WISHART, 2007).

Através destas bases de dados é possível construir perfis de potencial de atividades biológicas a partir de fragmentos proteicos (MINKIEWICZ et al., 2008). Sendo assim, a digestão proteica é essencial para o conhecimento das sequências peptídicas, pois os resíduos de determinados aminoácidos podem fornecer a indicação de sua bioatividade (FREITAS et al., 2013). Portanto, a utilização destas ferramentas após digestão proteica, tem sido considerada uma importante alternativa para se obter informações necessárias para a predição de bioatividade, para posterior síntese peptídica e pesquisas adicionais de interesse farmacológico e nutracêutico.

Diante do exposto, apesar da existência de estudos demonstrando a bioatividade da proteína dioscorina isolada de *D. cayennensis*, pesquisas envolvendo o conjunto das

proteínas deste tubérculo, sua digestão gastrointestinal simulada, bem como a predição *in sílico* da bioatividade dos peptídeos gerados e atividades biológicas *in vitro* não foram realizadas, sendo, portanto, um estudo inédito na literatura e que justifica a realização da presente pesquisa.

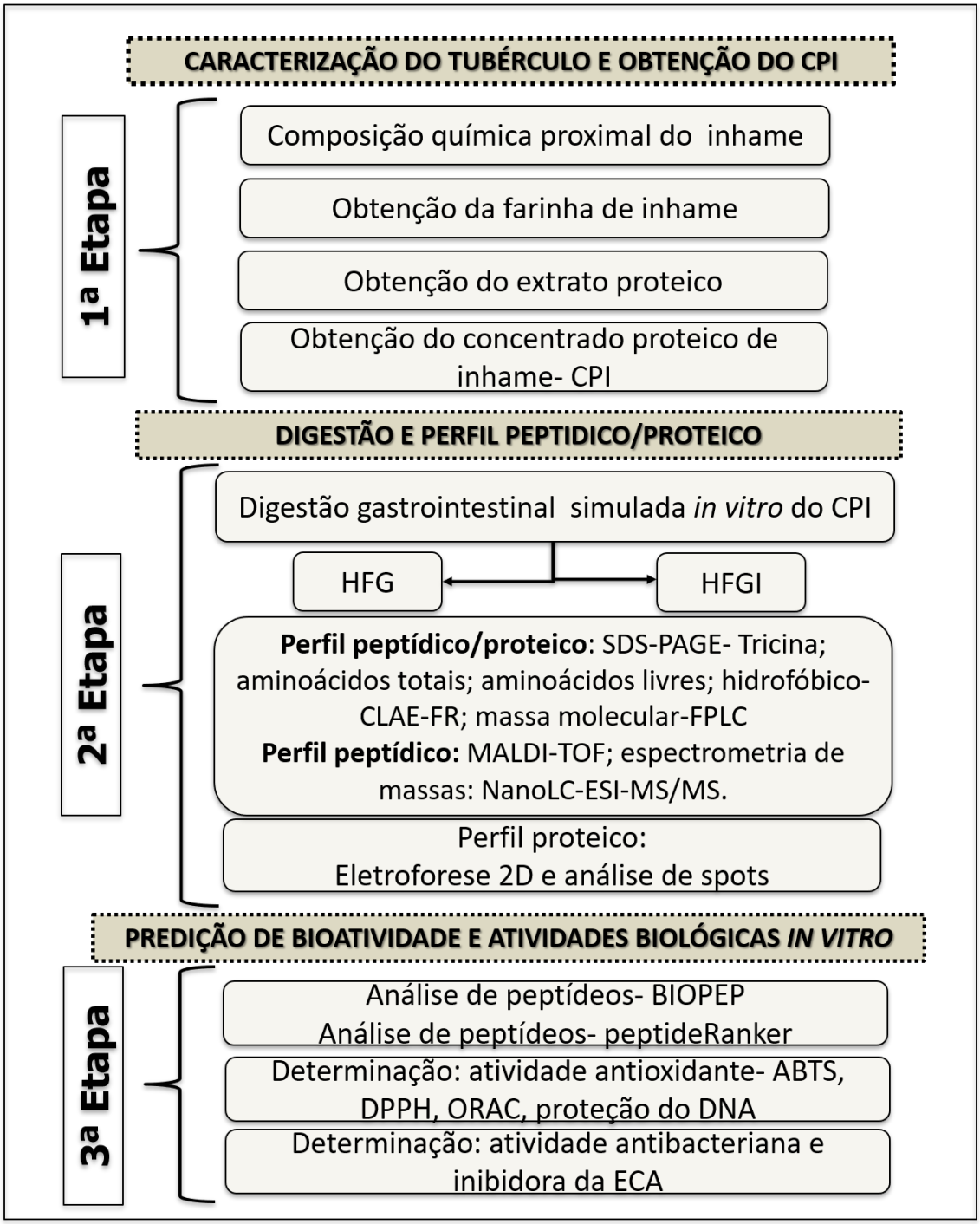
3 MATERIAL E MÉTODOS

3.1 LOCAL DE EXECUÇÃO E PLANEJAMENTO DA PESQUISA

Este trabalho foi desenvolvido nos Laboratórios de Proteômica Estrutural (LAPROTE), Laboratório de Bioquímica, Genética e Radiologia (BioGeR-LAB) da Universidade Federal da Paraíba (UFPB), e em parceria com instituições colaboradoras: Instituto de Tecnologia de alimentos (ITAL); Laboratório de Análises de Alimentos II (LAA II) e Laboratório de Virologia do Departamento de Genética e Biologia Molecular, ambos da Universidade Estadual de Campinas (UNICAMP), Campinas/SP e Laboratório de Proteômica da Universidade Federal de São Paulo-UNIFESP, São Paulo/SP.

A pesquisa foi realizada em três etapas: inicialmente, a amostra obtida foi submetida a composição química proximal, seguida da obtenção da farinha para o preparo do extrato proteico e por fim, do concentrado proteico de inhame (CPI). Na segunda fase o CPI foi submetido à digestão gastrointestinal simulada *in vitro*, gerando duas fases: hidrolisado fase gástrica (HFG) e hidrólise contínua até a obtenção do hidrolisado fase gastrointestinal (HFGI). O CPI ao perfil proteico (eletroforese-2D), as amostras: CPI, HFG e HFGI foram submetidas às análises de perfil proteico/peptídico (aminoácidos totais e livres, peso molecular por cromatografia de exclusão molecular, hidrofobicidade/hidrofilicidade e eletroforese SDS-Tricina). Para os hidrolisados foi realizado o perfil peptídico (nanoLC-ESI-MS/MS, MALDI-TOF). Na terceira etapa da pesquisa foi realizada análise de predição de bioatividade dos peptídeos através da base de dados BIOPEP e *Peptide Ranker* (predição de peptídeos inibidores da ECA). Foram realizadas análises de determinação de atividades biológicas *in vitro*: antioxidante, antibacteriana e inibidora da ECA. O Planejamento da pesquisa está simplificado na Figura 3.

Figura 3. Etapas do planejamento da pesquisa



Fonte: Autoria própria (2020)

3.2 ORIGEM E OBTENÇÃO DA AMOSTRA

Para o desenvolvimento deste estudo, o inhame (*Dioscorea cayennensis*) foi cultivado no período de fevereiro a setembro de 2015, na estação experimental de plantio da UFPB, campus II, localizada na microrregião do Brejo paraibano, no município de Areia/PB, Brasil. A região apresenta altitude de 574,62 m acima do nível do mar; longitude 35º 42' WGR e latitude 6º 58'12"S. Na classificação bioclimática de Gaussem, o bioclima predominante na área é o 3dth nordestino sub-seco, com precipitação pluviométrica média anual em torno de 1.400 mm. De acordo com a classificação de Köppen, o clima é do tipo As‟, que se caracteriza como sendo quente e úmido, com chuvas de outono-inverno (BRASIL, 1972) e a temperatura média anual oscila entre 23 a 24 ºC, com variações mensais mínimas.

A coleta do inhame foi realizada aos sete meses após o plantio, em setembro de 2015. Foram selecionados tubérculos com integridade física, de tamanho similar, com peso entre 500 g e 700 g. Os tubérculos foram transportados em temperatura ambiente (25±1 °C), dentro de sacos plásticos, para o Laboratório de Proteômica Estrutural (LAPROTE), campus I, UFPB/PB.

3.3 PREPARO DOS TUBÉRCULOS E DETERMINAÇÃO DA COMPOSIÇÃO QUÍMICA PROXIMAL

Os tubérculos foram lavados em água corrente para retirada da sujeira e posteriormente imersos em solução clorada a 200 ppm por 15 min., sendo em seguida lavados em água corrente para retirada do resíduo da solução. Após a assepsia, os inhames foram colocados à sombra para secagem. Em seguida, a amostra foi descascada, cortada e triturada para ser submetida a análise de caracterização química proximal, através das determinações de umidade, proteína total, cinzas e lipídeos, de acordo com a metodologia descrita por Latimer (2012), e os carboidratos totais por diferença dos demais constituintes. O conteúdo proteico total de proteínas foi mensurado pelo método de Kjedahl, multiplicando-se a porcentagem de nitrogênio pelo fator 5,75. Todas as análises foram realizadas em triplicata. A composição química proximal do tubérculo encontra-se descrita na Tabela 1.

Tabela 1. Composição química proximal do inhame (*Dioscorea cayennensis*).

Componentes	% (base úmida)	% (base seca)
Umidade	68±0,02	-
Cinzas	1,4±0,03	4,2
Proteína total	3,3±0,02	10
Lipídeos	0,4±0,01	1,2
Carboidratos*	26,9±0,02	84,6

* Carboidratos por diferença (100 menos o somatório dos outros componentes).
Resultados das análises com média de três repetições (± desvio padrão).

3.4 PREPARO DA AMOSTRA E OBTENÇÃO DO CONCENTRADO PROTEICO

Os inhames limpos foram descascados e cortados em pequenos pedaços. Porções de 100g foram trituradas em liquidificador com 100 mL de água destilada por 5 minutos. Este processo foi repetido e no final, todas as porções foram misturadas e homogeneizadas. A mistura homogeneizada foi submetida à filtração manual em malha de poliéster (40x40cm) para separação da mucilagem e amido, conforme metodologia descrita por Fonseca (2006) e apresentada na Figura 4.

Figura 4: Extração da mucilagem e amido do tubérculo inhame (*D. cayennensis*) por filtração manual em malha de poliéster

Fonte: Arquivo pessoal (2020)

Após a filtração em malha, o filtrado (amido e mucilagem) foi descartado e a massa do inhame retida na malha de poliéster foi recuperada. Esta massa foi espalhada em bandeja de plástico, para posterior adição de álcool etílico a 70% até sua completa imersão. Essa mistura foi deixada *overnight* em temperatura ambiente (18±1 °C) para retirada dos compostos fenólicos. A amostra em álcool foi centrifugada a 3372 x g, por 15 minutos e na sequência o sobrenadante foi descartado, e a massa de inhame foi colocada em bandeja de plástico à sombra, para evaporação do resíduo de álcool etílico. Por fim, após a secagem, a massa foi triturada em moinho tipo Whiley para obtenção de farinha fina.

Para a extração proteica, 100 g da farinha foi dissolvida em 1.000 mL de água destilada m/v (1:10), com ajuste de pH até 9,0 (NaOH 1 mol/L). Em seguida a mistura foi colocada em agitador magnético por 3 horas em temperatura ambiente (18±1 °C). A suspensão obtida foi centrifugada, em centrífuga modelo RC5C (Sorvall Instruments Dupont, Wilmingtin, EUA) sob refrigeração a 4 °C, a 3372 x g por 30 minutos. O sobrenadante foi recuperado e filtrado em papel filtro e seu volume foi medido. O precipitado foi utilizado para mais uma etapa de extração, utilizando-se o mesmo procedimento descrito acima. Os dois extratos resultantes foram misturados e denominado como extrato bruto total (EBT). Uma alíquota de 1mL foi retirada para determinar a quantificação de proteínas solúveis totais, pelo método de Bradford (1976), para o acompanhamento da eficiência da extração proteica.

O EBT foi submetido a precipitação das proteínas pelo ponto isoelétrico (PI) com ajuste do pH para 5,7 (HCl 1mol/L), e a recuperação proteica foi realizada por centrifugação a 4 °C, a 3372 x g por 20 min. O sobrenadante foi recuperado para repetição do processo de precipitação, com redução para o pH 4,5 (HCl 1mol/L), e a recuperação proteica foi realizada por centrifugação a 4 °C, a 3372 x g por 20 min. Os precipitados foram misturados e neutralizados até pH 7,0 usando NaOH 1 mol/L. Na sequência, o precipitado foi dialisado em membrana de celulose 3,5 kDa (Sigma Aldrich Co., St Louis, EUA) contra água Milli-Q em agitação, com 10 trocas de água a cada hora, em temperatura ambiente (18 ±1 °C). Em seguida, o precipitado foi liofilizado (modelo LS3000 Terroni) para obtenção do concentrado proteico de inhame (CPI). O CPI foi acondicionado em saco plástico e mantido a -18 °C para ser utilizado em análises posteriores. O CPI foi submetido a quantificação de proteína total pelo método de

Kjedahl, multiplicando-se a porcentagem de nitrogênio pelo fator 5,75 (LATIMER, 2012).

3.5 DIGESTÃO GASTROINTESTINAL SIMULADA *IN VITRO*

O CPI foi submetido ao processo de digestão gastrointestinal simulada *in vitro* de acordo com o protocolo harmonizado (INFOGEST, 2014; MINEKUS, 2014). Para o processo, 500 mg de CPI foi dissolvido em 2 mL de água deionizada a 37 °C e deixado em banho ultrassônico por 30 min. A solução foi submetida à ajuste do pH para 2,8 (HCl 6 mol/L), colocada em banho-maria a 37 °C para adição de 1 mL de enzima pepsina (20 mg/mL) ao meio reacional sob agitação. Após 120 min., a reação foi interrompida pela mudança de pH para 8,0 e reservou-se uma alíquota de 1 mL para análises posteriores. Em seguida, foi adicionado 1 mL de enzima (pancreatina 40 mg/mL) e sais biliares (24 mg/mL) sob agitação e mesma temperatura descrita acima, por 120 min. A digestão foi interrompida por aquecimento do sistema a 85 ºC por 10 min., seguido de imersão em banho de gelo.

O produto da digestão foi centrifugado a 3372 x g, por 15 minutos em centrífuga modelo RC5C (Sorvall Instruments Dupont, Wilmingtin, EUA). Os hidrolisados das fases gástrica (HFG) e gastrointestinal (HFGI) foram dialisados em membrana de 500 Da (Spectrum™ Labs Spectra/Por™, New Brunswick, NJ, USA) contra água deionizada por 10 vezes, em temperatura de refrigeração a 5±1 °C. Os dialisados foram recuperados, congelados e liofilizados em liofilizador Edwards – super Modulyo (West Sussex, RU).

3.6 GRAU DE HIDRÓLISE (GH)

A determinação do grau de hidrólise (GH) das fases HFG e HFGI foi realizada de acordo com Nielsen; Pertersen; Dambmann (2001). Preparou-se uma solução de cada amostra na concentração de 1 mg/mL, 400µL desta solução foi adicionada à 3 mL de o-phthaldialdehyde (OPA) 0,006 mol/L (contendo 1% SDS, DTT 0,0057 mol/L e tetraborato de disódio 0,17 mol/L). A mistura foi agitada durante 5 segundos em vórtex e após 2 min. em repouso, procedeu-se à leitura no comprimento de onda de 340 nm. No

ensaio foi utilizada a serina (0,1 mg/mL), como controle e o branco foi analisado com a mesma quantidade de água deionizada. O cálculo para o GH foi realizado de acordo com a expressão:

Equação 1:

$$DH\ (\%) = \frac{[(Abs_{sample} - Abs_{blank}) \div (Abs_{serine} - Abs_{blank})] \times [(\alpha \times 10) \div (m \times N) - \beta]}{h_{tot}} \times 100$$

Sendo: α é serina-NH2 meqv (0,9516), m é a massa da amostra, N é o fator de conversão de nitrogênio em proteína (5,75), β e htot são constantes definidas para diferentes matérias-primas de proteína (a referência de soja foi usada, aplicando-se 0342 e 7,8 como β e htot, respectivamente).

3.7 CARACTERIZAÇÃO DAS AMOSTRAS OBTIDAS

3.7.1 Composição química proximal

Para as análises da composição química proximal da farinha do inhame e CPI foram realizadas as determinações de umidade, proteína total, cinzas e lipídeos segundo metodologias descritas por Latimer (2012). O conteúdo de carboidratos totais foi quantificado diferença. O teor proteico total foi mensurado pelo método de Kjedahl, multiplicando-se a porcentagem de nitrogênio pelo fator 5,75.

3.7.2 Determinação do perfil de aminoácidos totais

A determinação de aminoácidos totais das amostras (CPI e HFGI) foi realizada em cromatógrafo líquido (Shimadzu Corporation, Tóquio, Japão) com uma coluna de fase reversa Luna C18 (250 mm x 4,6 mm, 5 µm, Phenomenex Inc., Torrence, CA, EUA). Os aminoácidos foram quantificados por comparação com os padrões de aminoácidos da Thermo Scientific (Rockford, EUA). Foi utilizado um padrão interno de ácido α-aminobutírico da Sigma-Aldrich® (St. Louis, MO, EUA). Medidas de aminoácidos totais

foram realizadas de acordo com os métodos relatados por Hagen, Frost e Augustin (1989). A determinação do conteúdo de triptofano foi realizada após análise enzimática com Pronase (40 °C/22-24 h), seguida de reação colorimétrica com 4- (dimetilamino) benzaldeído (DAB) em H_2SO_4 5 a 10,55 mol/L e leitura a 590 nm. O conteúdo de triptofano foi calculado a partir de uma curva padrão de L-triptofano (SPIES, 1967).

3.7.3 Determinação do perfil de aminoácidos livres

Os aminoácidos livres das amostras (CPI e HFGI) foram extraídos com HCl (ácido clorídrico) 0,1 mol/L (g mL^1) mediante a agitação orbital por 60 min., seguido de derivação em pré-coluna com fenilisotiocianato (PITC), de acordo com White, Hart e Fry (1986) e Hagen, Frost e Augustin (1989). A separação dos derivativos feniltiocarbamil-aminoácidos (PTC-aa) foi realizada em em sistema de cromatografia líquida de alta resolução (Shimadzu Corporation, Tokyo, Japan) em coluna de fase reversa C18 - Luna - Phenomenex (250 mm x 4,6 mm, 5 µm; Phenomenex Inc., Torrence, CA, USA). A injeção da amostra foi efetuada automaticamente (50 µL) e a detecção ocorreu a 254 nm. A separação cromatográfica foi realizada a um fluxo constante de 1 mL/min., à temperatura de 35 ºC. O tempo de corrida cromatográfica foi de 45 minutos e os resultados foram expressos em mg de aminoácido por 100 g de amostra. A quantificação foi realizada mediante a adição do padrão interno ácido α-aminobutírico e a identificação dos aminoácidos foi realizada por comparação com o padrão externo (Pierce, PN 20088).

3.7.4 SDS-PAGE-Tricina

O processo de hidrólise foi acompanhado por eletroforese em gel de poliacrilamida em tricina (SCHAGGER; JAGOW, 1987). Para o sistema de SDS-PAGE/Tricina foram utilizados três géis: de empilhamento (4% T e 3% C), espaçador (10% T e 3% C) e separação (16% T e 3% C). As amostras foram diluídas em tampão redutor, homogeinizadas em vórtex, em seguida foram colocadas em estufa a 100 °C por 10 min. As amostras foram aplicadas ao gel, a corrida do gel ocorreu a 25 mA, por aproximadamente 6 horas. Após o tempo de corrida, o gel foi fixado em solução fixadora

de metanol, ácido acético e água (5:1:4 v/v/v), em seguida o gel foi corado em solução de 0,025% de Coomassie Brilhant Blue G-250 em ácido acético 10%.

Para comparação dos pesos moleculares das amostras foi utilizado marcador padrão de baixo peso molecular (Low Range Molecular Weight Marker da GE Healthcare Life Science, Nova Jersey, EUA) com bandas na faixa de 3,5 a 38 kDa.

3.7.5 Eletroforese bidimensional

A análise do perfil eletroforético em SDS-PAGE 2D do CPI foi realizada de acordo com O'Farrell (1975), em um sistema Protean 2-D (Bio-Rad). Para separação pela massa e carga em duas etapas: focalização isoelétrica e eletroforese-SDS em gel de poliacrilamida em concentração de 15% a 300V. Para a focalização isoelétrica foi realizada faixa de pH gradiente 3.5 a 11.0. A visualização dos componentes proteicos nos géis foi realizada por coloração com a Solução de Coloração Brilliant Blue R (Sigma, EUA). O mapeamento dos spots de proteínas foi feito com o sistema Scanner de géis ImageScan da GE Lifesciences. Os mapas de proteínas foram analisados usando o programa ImageMaster 2D Platinum v.7 (GE Healthcare, UK).

3.7.6 Perfil de distribuição de massa molecular

As amostras CPI, HFG e HFGI foram caracterizadas quanto ao perfil de distribuição de massa molecular por cromatografia líquida de exclusão molecular, em sistema de cromatografia FPLC (Akta Pure, GE Healthcare, Chicago, Illinois, EUA), com detecção fixada em 280 nm e coluna cromatográfica Superdex 30 Increase 10/300 GL (GE Healthcare, Chicago, Illinois, EUA) (faixa de separação 100-7000 Da). O monitoramento e obtenção dos cromatogramas foram feitos pelo Software Unicorn 6.3. As amostras (concentrações variáveis) ou padrões (0,2 e 1,0 mg/mL) foram solubilizados em fase móvel (tampão fosfato de sódio 0,025 c, em pH 7,4, com NaCl 0,15 mol/L) e sonicados por 10 min. Tanto a fase móvel quanto as amostras ou padrões foram filtradas em membrana hidrofílica de politetrafluoretileno (PTFE; 0,45 µm). O volume de injeção de amostra foi de 100 µL e o tempo de corrida igual a 65 min. Foram utilizados os padrões α-Lactoalbumina (14178 Da), Insulina (5807,6 Da), Vitamina B12 (1355,4 Da) e L-β-4-

Dihidroxifenilanina (197,2 Da) para construção da curva analítica (log MM x TR) e o cálculo da porcentagem de distribuição de massa molecular (MM) de cada faixa foi identificada com seu tempo de retenção (TR).

3.7.7. **Perfil de hidrofilicidade**

O perfil de hidrofilicidade das amostras (CPI, HFG e HFGI) foi determinado por cromatografia líquida de alta eficiência em fase reversa (CLAE-FR), em sistema de HPLC Shimadzu, com detector photodiode array (PDA) (Shimadzu, Japão), coluna C18 Luna 100 Å (4,6 mm x 250 mm; partícula de 5 μm) (Phenomenex, CA, EUA). A composição dos solventes foi: solvente A- água Milli-Q com 0,1% de ácido trifluoracético (TFA); solvente B- acetonitrila com 0,1% TFA, filtrados em membrana hidrofílica de politetrafluoretileno (PTFE; 0,45 μm). A coluna foi mantida em temperatura ambiente, com fluxo de 1 mL/min., com detecção em 214 nm, volume de injeção de 50 μL, e o tempo de corrida foi de 55 min. As amostras (3 mg proteína/mL para hidrolisados e 1mg/mL para CPI íntegro) foram eluídas em gradiente linear de 5 a 20% do solvente B em 20 min, chegando a 40% de solvente B em mais 20 min e até 80% nos 10 minutos seguintes. Nos últimos 5 min. de corrida, retornou-se à condição de 5% de solvente B.

3.8 ANÁLISE POR DESSORÇÃO/IONIZAÇÃO A LASER ASSISTIDA POR MATRIZ - TEMPO DE VOO - (MALDI-TOF-MS)

A análise de MALDI-TOF-MS foi realizada para avaliação do perfil de massa molecular de peptídeos obtidos a partir de digestão gastrointestinal simulada das proteínas do inhame. A mistura peptídica (HFG e HFGI) seca foi diluída em ddH2O/ácido trifluoroacético (TFA) 0,1%, filtrada em filtro de nylon de 0,22 μm e 1 uL de solução peptídica foi misturada (1:1) com uma solução de ácido α-ciano-4-hidroxicinâmico (α Matriz -CHCA, 25 mg/mL) em 0,1% v / v TFA, 30% v/v acetonitrila. A alíquota de 1 μL da mistura de peptídeos foi colocada em um alvo de amostra de aço moído MSP 96 (Bruker Daltonik, Bremen, Alemanha) e seco à temperatura ambiente. Foram preparados quatro pontos para análise.

Os espectros MALDI-TOF-MS foram adquiridos com o espectrômetro de massa linear Microflex LT (Bruker Daltonics), usando o pacote de software FlexControl (versão

3.4, Bruker Daltonics). Os espectros foram registrados no modo linear positivo (frequência do laser, 1000 Hz; tensão da fonte de íons 1, 20,05 kV; tensão da fonte de íons 2, 18,35 kV; tensão da lente, 6,22 kV; taxa de amostragem, 0,50 GS/s; extração de íons pulsados, 230 ns; fator de ganho, 15,1x) e cinco espectros independentes (1000 disparos em posições aleatórias no mesmo local de destino, para espectro) foram coletados manualmente. As medições foram realizadas em análises independentes nas faixas de massa 500-1520 Da, 1500-2600 Da, 2500-3600 Da calibradas externamente usando o Peptide Calibration Standard 2 (Bruker Daltonics) de acordo com as especificações da faixa de massa.

3.9 ANÁLISE DE IDENTIFICAÇÃO DOS PEPTÍDEOS- nanoLC-ESI-MS/MS

As misturas de peptídeos obtidas de cada hidrolisado proteico (HFG e HFGI) foram ressuspensas em solução aquosa de acetonitrila a 2%/ácido fórmico a 0,1% e submetidas à análise por cromatografia líquida em nano fluxo acoplada à espectrometria de massas sequencial, com ionização por *Electrospray* (nanoLC-ESI-MS/MS), realizada em sistema NanoLC Dionex Ultimate 3000 (Thermo Fisher Scientific), acoplado a um espectrômetro de massa Impact II quadrupolo tempo-de-voo (Q-TOF) (Bruker Daltonics). Os peptídeos foram retidos na coluna Acclaim Pepmap nano-trap (Dionex-C18, 100 Å, 75 μm x 2 cm) e separados *on-line* usando a coluna analítica Acclaim Pepmap RSLC (Dionex-C18, 100 Å, 75 μm × 15 cm) sob gradiente eluição de 2 a 98 % (v/v) de acetonitrila 0,1 % de ácido trifluoroacetico durante 180 min, e o fluxo ajustado para 300 nL/min. Os espectros de massa foram adquiridos no modo íon positivo com os precursores MS e os produtos MS/MS adquiridos a 2 Hz na gama de massas de 50-3000 m/z e os parâmetros de energia de dissociação induzida por colisão ramificada (CID) variaram de 7 a 70 eV.

3.9.1 Análise de bioinformática

Os arquivos de dados brutos do MS (arquivo) contendo espectros MS/MS foram importados no software PEAKS Studio 8.5 (Bioinformática Solution Inc., Waterloo,

Canadá) para análise de novo e pesquisas de banco de dados (ZHANG et al., 2012). Os seguintes parâmetros foram usados: tolerância de massa precursora de 10 ppm; tolerância de massa de fragmento do íon de 0,025 Da; sem clivagem enzimática específica; oxidação em Metionina (+15.99 Da) e Pyro-glutamico a partir da glutamina (-17.03 Da), como modificações variáveis. Como o banco de dados de *Dioscorea cayennensis* apresentou poucas sequências proteicas, foram selecionados peptídeos *de novo* com média de confiança local (*Average local confidence*, ALC) ≥ 50% e submetidos à busca no banco de dados utilizando ferramentas SPIDER (HAN et al., 2005), contra o banco de dados Dioscorea da Uniprot KB (71 sequências de Swis-prot e 2703 sequências de TrEMBL, baixado em 03 de maio de 2018 de http://www.uniprot.org/). As taxas de falsos positivos (*false discovery rate*, FDR) foram fixadas em no máximo 1%, correspondente aos espectros de MSMS atribuídos aos peptídeos (*Peptide Spectrum-Matches*, PSM).

3.10 ANÁLISE DE PREDIÇÃO DA BIOATIVIDADE DOS PEPTÍDEOS

A predição da bioatividade dos peptídeos identificados a partir da análise LC-ESI /MS/MS foi realizada submetendo as sequências à análise na base de dados BIOPEP (disponível em http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) (MINKIEWICZ et al., 2008). As sequências de cada peptídeo com predição para inibidor de ECA, foram submetidas ao servidor Peptide Ranker (http://bioware.ucd.ie/~compass/biowareweb/Server_pages/peptideranker.php), para inferir a probabilidade de sua bioatividade (PIOVESANA et al., 2015).

3.11 ATIVIDADES BIOLÓGICAS

3.11.1 Determinação da atividade antioxidante

3.11.1.1 Análise da capacidade sequestrante do radical DPPH

A capacidade sequestrante do radical 2,2-difenil-1-picrilidrazil **(**DPPH) foi determinada de acordo com a metodologia descrita por Picot et al. (2010), com ligeiras modificações. Alíquotas de 30 µl de amostra CPI, HFG e HFGI (10- 20 mg/mL) foram diluídas em água destilada e misturadas à 1,5 mL de solução de DPPH (60 µmol/L) diluído em metanol P.A. A mistura foi agitada durante 60 min., a 25 ◦C e na ausência de luz. Em seguida, a amostra foi centrifugada a 14.500 rpm durante 5 min. (centrífuga Sigma® 2K15) e a absorbância do sobrenadante foi mensurada a 517 nm. A concentração do radical DPPH nas amostras foi mensurada a partir de uma curva de calibração, usando Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), (500 - 2.000 µmol/L). A capacidade das amostras sequestrarem o DPPH foi expressa como a percentagem do efeito do Trolox sobre o radical livre na Equação 2:

% Efeito sequestrante = [(Abs branco – Abs amostra) / Abs branco] × 100 (Eq.2)

Adicionalmente, o coeficiente de capacidade antioxidante equivalente de Trolox (TEAC) foi calculado dividindo a concentração de Trolox pela curva e a concentração final da amostra em mg/mL.

3.11.1.2 Análise da capacidade sequestrante do radical ABTS

A capacidade sequestrante do radical 2,2’-azinobis- (3-ethylbenzothiazoline)-6-sulfonic acid (ABTS•+), pelo CPI, HFG e HFGI foi determinada de acordo com a metodologia descrita por Wiriyaphan; Chitsomboon; Yongsawadigul (2010). Uma solução estoque de ABTS•+ foi preparada por mistura da solução de ABTS•+ (7 µmol/L) e persulfato de potássio (140 µmol/L), em seguida esta solução foi mantida ao abrigo da luz durante 16 h. A solução de trabalho foi elaborada diluindo a solução estoque em etanol P.A. para atingir a absorbância de de 0,7 ± 0,05 em 734 nm., usando Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (1.000 µmol/L), para construção da curva padrão. Alíquotas de 20 µL de amostra (10- 20 mg/mL) foram misturadas com 3,0 mL da solução do radical ABTS•+ em tubos de ensaio e homogeneizadas em agitador de tubos. Após 6 min. a leitura foi realizada a 734 nm. A capacidade de eliminação do radical ABTS•+ foi calculada de acordo com a Equação 3:

Capacidade de eliminação de radicais ABTS (%) = [(Abs branco- Abs amostra/ Abs do branco) x 100] (Eq.3)

O coeficiente de capacidade antioxidante equivalente de Trolox (TEAC) foi calculado dividindo a concentração de Trolox pela curva e a concentração final da amostra em mg/mL.

3.11.1.3 Análise da capacidade de absorção de radicais de oxigênio- ORAC

A determinação do ORAC foi realizada de acordo com o método descrito por Huang et al. (2002). A reação foi realizada em tampão fosfato 0,075 mol/L (pH 7,4). As soluções contendo as amostras CPI, HFG e HFGI (25 µl) e fluoresceína (150 µl, 55 nM) foram adicionados em microplaca de 96 poços e a reação foi iniciada pela adição de 30 µl de AAPH (25 nM). A intensidade da fluorescência foi medida por 50 min., a cada 60 segundos durante 50 ciclos, com comprimentos de onda de excitação e emissão de 485 nm e 520 nm, respectivamente, a 37 °C em leitor de microplacas (Enspire 2300, Leitor de Placas Multimodo, Perkin Elmer, EUA). Foi utilizado Trolox (0,05 - 4,0 µM) como controle positivo e tampão fosfato como controle negativo. ORAC foi definido como equivalente de trolox (mmol TE/g amostra ou mmol TE/mg de amostra) de acordo com a área sob a curva (AUC).

3.11.1.4 Ensaio de proteção de danos ao DNA

O teste de proteção contra danos causados ao DNA foi realizado utilizando o CPI e hirolisados, de acordo com Huang et al. (2010), com algumas modificações. O radical hidroxilo (•OH) foi gerado pela reação de Fenton de acordo com o método de Kohno et al. (1991). Alíquotas de 15 µL da mistura reacional contendo CPI, HFG e HFGI (10 e 15 mg/mL), 5 µL de DNA plasmídico de *Escherichia coli* (1 mg/mL), 2 µL de FeSO4 0,018 mol/L e 3 µL de hidrogénio 60 mol/L. O peróxido foi incubado a 37 °C durante 30 min. Em seguida, foi adicionado 2 µL de EDTA 0,001 mol/L para interromper a reação. O

teste em branco continha apenas DNA de plasmídeo e o teste de controle continha todos os componentes da reação e água. A mistura foi então submetida à electroforese em gel de agarose a 1%. Finalmente, as bandas de DNA foram coradas com Gel-Red™ (Biotium, Inc., Hayward, EUA) e fotografadas por um Transluminator L.Pix Loccus-Molecular Imaging.

3.11.2 Avaliação da atividade inibitória da ECA

Para a análise da atividade anti-hipertensiva das amostras CPI, HFG e HFGI, foi utilizado pulmão de coelho como fonte de enzima conversora de angiotensina (ECA) (Sigma, 10386). A atividade de ECA foi realizada com Abz-FRK(Dnp)P-OH 0,010 mol/L e foi medida continuamente a 37 ºC em espectrofluorímetro de PC Shimadzu RF-1501 PC ajustado para λex = 320 nm e λem = 420 nm. Os ensaios foram realizados em tampão Tris-HCl a 0,1 mol/L, pH 7,0, contendo NaCl 0,05 mol/L e $ZnCl_2$ 0,00001 mol/L. Ensaios de inibição foram realizados sob as mesmas condições após 1 min. de pré-incubação das enzimas com concentrações crescentes das amostras (FARIAS et al., 2006). A emissão de fluorescência foi medida continuamente e os valores da metade da concentração inibitória máxima (IC_{50}) foram obtidos usando a equação $y = 100\%/1+(x / IC_{50})^S$, onde S é um fator de inclinação.

Os valores de IC_{50} para o inibidor de ECA foram calculados pelo programa de análise de dados de titulação de ligação Grafit (LEATERBARROW, 2001).

3.11.3 Avaliação de atividade antibacteriana

A atividade antibacteriana do CPI, HFG e HFGI foi testada segundo as normas do protocolo M7-A6 do *National Comite For Clinical Laboratory Standards* – NCCLS (2003), por meio da técnica de microdiluição em caldo, com as linhagens *Escherichia coli*, *Salmonella* sp e *L. monocytogenes*. Cada cepa, armazenada a 4 °C, foi colocada para crescer em meio *Brain Heart Infusion* (BHI), a 35 °C, até se obter aproximadamente $1,5\times10^8$ UFC/mL, escala de *Mc Farland* de 0,5. Para o teste antimicrobiano foram usadas microplacas de *Enzyme Linked Immuno Sorbent Assay* (ELISA) de fundo chato com 96

poços. Foi colocado 90 µL de meio em cada poço, em seguida, foram distribuídos 90 µL da amostra (1 mg/mL) e, desta mistura, foram feitas diluições seriadas em triplicata. Em seguida, foram adicionados 10 µL de suspensão bacteriana (10^6 UFC/mL) em cada poço contendo a amostra diluída no meio. O controle negativo abrangeu apenas o meio, e o positivo, bactéria e o meio. A placa foi incubada a 35 °C em espectrofotômetro (*MultiskanTM GO Microplate Spectrophotometer*, Thermo Scientific), e o crescimento bacteriano foi monitorizado pela medida da absorbância a 625 nm, a cada 60 min, durante 24 h.

3.12 ANÁLISE ESTATÍSTICA

Para análise estatística foi utilizado o programa de software *GraphPad* versão 6.0. Os resultados foram expressos em média ± DP. Foi utilizado o teste ANOVA, seguido de Teste t de Student. Diferenças estatisticamente significativas foram consideradas quando $p < 0,05$.

A análise *de novo* foi realizada com tolerância de massa precursora de 07 ppm, tolerância de massa de fragmento de 0,025 Da, nenhuma clivagem enzimática específica foi estabelecida como modificações variáveis de oxidação em Metionina (+15.99 Da) e Pyro-glu de Q (-17.03 Da). Como o banco de dados de *D. cayennensis* apresentou poucas sequências proteicas, foram selecionados peptídeos sequenciados *de novo* com média de confiança local (ALC) ≥50% e submetidos à busca no banco de dados utilizando ferramentas SPIDER (Han et al., 2005), contra o banco de dados Dioscorea da Uniprot KB (71 sequências de Swiis-prot e 2703 sequências de TrEMBL, baixado em 03 de maio de 2018 de http://www.uniprot.org/). As taxas de descobertas falsas (FDRs) para proteínas e peptídeos foram fixadas em no máximo 1%. Peptídeos com ALC> 90% sem pareamento no banco de dados de homologia foram considerados como análise complementar.

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4 RESULTADOS E DISCUSSÃO

Os resultados obtidos nesta tese estão apresentados em forma de artigos científicos submetidos em periódicos de elevado impacto na área de Ciência e Tecnologia de Alimentos.

4.1 ARTIGO I: Submetido ao periódico *International Journal of Biological Macromolecules*

Yam (*Dioscorea cayennensis*) protein concentrate: Production, characterization and evaluation of digestibility *in vitro*

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Abstract

The aim of this study was to produce a yam protein concentrate (YPC), *Dioscorea cayennensis,* to evaluate its protein digestibility, as well as the peptide profiles generated through the simulated gastrointestinal digestion (GID) *in vitro.* In this study was obtained a YPC that resulted in 64.0 % protein. In order to evaluate its digestibility, simulated GID was perfomed *in vitro,* as well as degree of hydrolysis and free amino acids. For the peptide profile it was used mass spectrometry (MS) through nanoLC-ESI-MS/MS, essential amino acids, molecular weight (MW) profile and hydrophobicity profile. After the digestion, a significant release of free amino acids was noticed when compared to the YPC ($p < 0,5$). The effectiveness of the GID was verified according to molecular weight (MW) distribuction profile, with nearly 71% of the peptides with MW< 2 kDa produced in GID phase. Those results have shown that the final phase of GID was responsible for the production of smaller peptides. Most of the peptides belongs to the dioscorin protein from *D. cayennensis.* Peptides as KQAVNE**NAINNARPL**QPTN, **GRSDPFL**SDL, KNEINAG**VVDPN**QLQF identified in the gastric phase, had their MW decreased with the GID hydrolysis progression, resulting in **NAINNARPL**, **GRSDPF** and **VVDPN.** From these findings, the GID turns out to be efficient, producing molecules with smaller MW, suggesting that could occur the release of amino acids with the exhibition of ionizable and functional groups, potentializing the yam proteins bioactive capacity. Thus, the GID is essential to create a gamma of peptides. In its turn, the identification of these peptides makes it possible to perform the prediction of the bioactive role of these molecules in the biological process. Although, the peptides obtained after the digestion should be explored in future studies, aiming to know the bioactive potential of these molecules for human health.

Key-words: simulated gastrointestinal digestion *in vitro, Dioscorea cayennensis,* dioscorin, nanoLC-*ESI*-MS/MS.

1. Introduction

The Yam *(Dioscorea* spp.*)* belongs to the Dioscoreaceae family and it is considered as an important tuber cultivated in Africa, Asia, as well as South and Central America [1]. The tubers are part of human food culture for many centuries. The most important edible species to be cultivated are: *Dioscorea opposite* Thunb (Chinese yam), *D. alata*, *D. japonica* (Japanese yam), *D. pseudojaponica*, *D. batatas* (Korea yam), and *D. cayenennsis* [2]. Nutritionally, the tubers of these species represent a substantial starch source, however a protein from *Dioscorea* genus has been gaining prominence, the dioscorin, its main storage protein [3]. The biochemistry description of the yam *D. cayenensis* proteins, showed that 85% of total protein is made of dioscorin A and B [4].

The benefits of this tuber are known and used in traditional chinese medicine, as phytotherapic treatment for diabetes, aging, neurodegenerative diseases and cancer. Studies from the last decade have shown that dioscorin and its hydrolysis and peptides are responsible for biological activities *in silico*, *in vitro* and *in vivo* [5, 6]. Although, data about the tubers *Dioscorea* proteins group, as well as their digestibility and bioavailability is yet scarce.

In the scope of food science, the study of digestion *in vitro* consists in an excellent resource of investigation to understand the quality of the food, as the behavior of its composition and structure during digestion [7]. This knowledge contributes to evince a better utilization of the nutrientes and diet formulation that keeps a nutritional balance. According to Lorieau et al. [8], after the knowledge about the amino acids composition, protein digestibility and bioavailability of the peptides and amino acids generated, are essential factors to identify the quality of food protein.

According to Sarmadi and Ismail [9], the fragmented proteins release specific amino acids sequences fragments after the digestion, that are capable to express biological activity. Thereby, simulated gastrointestinal digestion *in vitro* represents a viable alternative to peptides obtainment, focusing in the investigation of potential health benefits [10]. In this context, Minekus et al. [11] emphasizes the use of simulated gastrointestinal digestion (GID) *in vitro,* aiming to approach the digestibility and bioacessibility in several pharmaceutical products and macronutrients, like proteins.

In this context, the present study aimed to produce yam protein concentrate to evaluate its protein digestibility, as well as the peptide profiles generated through the simulated gastrointestinal digestion *in vitro.*

2. Material and methods

2.1. Materials

The yam tubers were acquired in an experimental planting at UFPB-Campus Areia (João Pessoa, PB, Brazil). Porcine pepsin (≥250 units / mg) and porcine pancreatin, as well as bile salts, were purchased from Sigma-Aldrich® (St. Louis, MO, USA), α-aminobutyric acid and tricine were also purchased from Sigma-Aldrich ® (St. Louis, MO, USA). [Glu1]-Fibrinopeptide, acetonitrile were purchased from Waters (Milford, MA, USA). A-Lactoalbumin (L6010), Insulin (I2643), L-β-4- Dihydroxyphenylanine (D-9628) and Vitamin B12 (Fw13554) were purchased from Sigma Aldrich (St. Louis, USA). Other solvents and chemical reagents used in this research, were all of analytical grade.

2.2 Preparation of yam flour

Yam tubers, after being sanitized, shade-dried and cut, were mixed in distilled water in a 1:10 ratio and grinded. After filtration in a polyester fabric, the recovered yam mass was left overnight in 70% ethyl alcohol. Then the mass was centrifuged at 3372 g for 15 min., with the supernatant discarded and the dry mass powdered to obtain the yam flour.

2.3. Preparation of yam protein concentrate

For protein extraction the yam flour was dispersed in distilled water (1:10, w/v) and the pH was adjusted to 9.0 with NaOH 1 mol/L The suspension was stirred for 3 h at 25 °C and centrifuged at 3372 × *g* during 30 min. at 4 °C. Supernatant was recovered and the precipitate was used to repeat the protein extraction process under the same conditions. The supernatants were combined, subjected to protein precipitation with pH adjustment to 5.7 (using HCl 1 mol/L) and centrifuged at 3372 × *g* during 20 min. at 4

°C. Supernatant was recovered to repeat the precipitation process, adjusting pH to 4.5 (using HCl 1 mol/L). The precipitates were combined and neutralized to pH 7.0 with NaOH 1 mol/L. The protein concentrate was dialyzed in Milli-Q water, under magnetic stirring at 25 °C, on a 3.5 kDa MWCO cellulose membrane (Spectrum™ Labs Spectra/Por™, New Brunswick, NJ, USA). Then, the protein concentrate was frozen and freeze-dried (freeze-dryer model LS3000, Terroni Equipamentos, SP, Brazil) to obtain the yam protein concentrate (YPC).

2.4. Protein content

The samples total protein content was measured by Kjedahl method, multiplying the percentage of nitrogen by 5.75 [12]. All analyzes were performed in triplicate.

2.5. Two-dimensional electrophoresis

The two-dimensional electrophoresis (2-DE) of the YPC was performed according to the methodology described by O'Farrel [13]. The sample (1 mg) was solubilized in a solution consisting of 7 mol/L urea, 2 mol/L thiourea, 0,5% Immobilized pH gradient (IPG) gel, dithiothreitol (DTT), 2% CHAPS and 0.002% bromophenol blue. The 13 cm non-linear strip, pH 3 to 11, was rehydrated in IPGbox for 18 h, at ± 25 °C (GEHealthcare, WI, USA). After that time, the strip was subjected to isoelectrofocusing (IPGPhor Healthcare® GE III). At the end of focusing, the strip was stored in a 1.5 mol/L Tris-HCl buffer solution, pH 8.8, with 1 mL of 10% SDS [14].

The strip was equilibrated in solution with DTT and iodacetamide. It was then transferred to the 15% polyacrylamide gel. The program run was performed at 100 V, 30 mA and 50 W. A 225 to 12 kDa marker (Amersham ECL Rainbow Molecular Full Range, GE Healthcare Life Sciences) was used. At the end of the run, the gel was fixed with acid solution acetic acid, 95% ethanol and milli-Q water (10/40/50), and stained with CoomassieBrilliant Blue G-250 2%, for 72 h. After destaining with methanol solution; acetic acid and distilled water (10/20/70), the gel was scanned on an Image Scanner III scanner (GE Healthcare Bio-Sciences, WI, USA), and the spots detected and identified by the ImageMasterTM2D Platinum 7.0 software (GE Healthcare Bio-Sciences, WI, USA).

2.6. In vitro simulated gastrointestinal digestion of YPC

The YPC digestion was performed following the internationally accepted digestion protocol developed by the INFOGEST Cost Action Minekus et al. [11]. Briefly, 500 mg of YPC were dissolved in 5 mL of deionized H_2O at 37 °C and left in an ultrasonic bath for 30 min. The mixture, previously submitted to pH 2.8 adjustment using HCl 1 mol/L, was added with porcine pepsin (20 mg/mL) and subjected to digestion for 120 min at 37 °C, in a water bath under agitation at 150 rpm (gastric phase). Samples were taken at the starting point and after 120 min of digestion. The gastric phase was interrupted by pH adjustment to 8.0 with NaOH 1 mol/L. The suspension from the gastric phase was mixed with the same volume of simulated intestinal fluid (pH 8.0) containing porcine pancreatin (40 mg/mL) and porcine bile extract (24 mg/mL) for 120 min at 37 °C, in a water bath under agitation at 150 rpm (total gastrointestinal phase). The digestion was stopped by heating at 85 ºC for 15 min., followed by centrifugation at 3700 g for 15 min (centrifuge model RC5C, Sorvall Instruments Dupont, Wilmington, USA). Digestions were performed in duplicate.

The hydrolyzates of the gastric phase (GPH) and total gastrointestinal phase (GIPH) were dialyzed in Milli-Q water in a 500 Da membrane (Spectrum™ Labs Spectra/Por™, New Brunswick, NJ, USA), recovered, frozen and freeze-dried (Edwards super Modulyo, West Sussex, UK) for further analysis.

2.7. Degree of hydrolysis (DH)

The degree of hydrolysis (DH) was determined according to Nielsen, Pertersen, Dambmann (2001), using 6 mM o-phthaldehyde (OPA) (containing 1% SDS, 5.7 mM DTT and 0.17 $mol.L^{-1}$ disodium tetraborate). The calibration curve was built with serine (0.1 $mg.mL^{-1}$). Deionized water was used as blank. DH calculation was performed according to the equation

$$DH\ (\%) = \frac{\left[\left(Abs_{sample} - Abs_{blank}\right) \div \left(Abs_{serine} - Abs_{blank}\right)\right] \times \left[(\alpha \times 10) \div (m \times N) - \beta\right]}{h_{tot}} \times 100$$

where α is serine-NH_2 meqv (0.9516), m is the sample mass, N is the nitrogen-to-protein conversion factor (5.75), β and h_{tot} are constants defined for different protein raw materials (soy reference was used, applying 0342 and 7.8 as β and h_{tot}, respectively).

2.8. Tricine-SDS-PAGE

The hydrolysis process was accompanied by tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) [16]. For the Tricine-SDS-PAGE system, three gels were used: stacking (4% T and 3% C), spacer (10% T and 3% C) and resolving (16% T and 3% C). The samples were diluted in reducing buffer, homogenized in a vortex, then placed in an oven at 100 °C for 10 min. The samples were applied to the gel, and run at 25 mA, for approximately 6 hours. After the running time, the gel was fixed in a solution of methanol, acetic acid and water (5:1:4 v/v/v) and stained in a solution of 0.025% Coomassie Brilliant Blue G-250 in 10% acetic acid. To compare the samples molecular weights, a standard low molecular weight marker (GE Healthcare Life Science, New Jersey, USA) of 38 to 3.5 kDa was used.

2.9. Total amino acid analysis

Total amino acid analysis was performed on a liquid chromatograph (Shimadzu Corporation, Tokyo, Japan) using a Luna C18 reverse phase column (250 mm x 4.6 mm, 5 µm, Phenomenex Inc., Torrence, CA, USA). Amino acids were quantified by comparison with ThermoScientific (Rockford, USA) amino acid standards. An internal standard of α-aminobutyric acid from Sigma-Aldrich® (St. Louis, MO, USA) was used, according to Hagen, Frost and Augustin [17]. Tryptophan content was determined after enzymatic analysis with Pronase (40 °C/22-24 h), followed by colorimetric reaction with 4- (dimethylamino) benzaldehyde in H2SO4 at 10.55 mol/L and reading at 590 nm. Tryptophan content was calculated from a standard L-tryptophan curve [18]. Amino acid score (AAS) and essential amino acid index (EAAI) were used to measure the proteins biological quality. These parameters were calculated according to Friedman [19] using the standard amino acid requirement FAO/WHO/ UNU [20]. Where EAAS = mg of EEA in 1g of test sample protein / mg of EAA in 1g of standard protein (FAO/WHO) × 100.

2.10. Analysis of free amino acids

Free amino acids were extracted with 0.1 mol/L hydrochloric acid (g/mL) using orbital agitation for 60 min, followed by derivation in a pre-column with phenylisothiocyanate (PITC), according to White, Hart and Fry [21] and Hagen, Frost and Augustin [17]. The separation of the phenylthiocarbamyl-amino acid derivatives (PTC-aa) was performed using a high-performance liquid chromatograph (Shimadzu Corporation, Tokyo, Japan) in a C18 - Luna - Phenomenex reverse phase column (250 mm x 4.6 mm, 5 µm; Phenomenex Inc., Torrence, CA, USA). The sample was injected automatically (50 µL) and the detection occurred at 254 nm. The chromatographic separation was carried out at a constant flow of 1 mL/min, at a temperature of 35 ºC. The chromatographic run time was 45 minutes and the results were expressed in mg of amino acid per 100 g of sample, in which quantification was performed by adding the internal α-aminobutyric acid standard and identification of the amino acids performed by comparison to the external standard amino acids from Thermo Scientific (Rockford, USA).

2.11. Molecular weight distribution

The YPC, gastric (GPH) and gastrointestinal (GIPH) phases hydrolyzates were characterized according to the molecular mass distribution profile by size exclusion liquid chromatography, in an FPLC chromatography system (AktaPure, GE Healthcare, Chicago, Illinois, USA) with detection at 280 nm in a Superdex 30 Increase 10/300 GL chromatographic column (GE Healthcare, Chicago, Illinois, USA), which separation range is 100-7000 Da. Chromatograms were monitored and obtained by the Unicorn 6.3 Software. The samples (varying concentrations) or standards (0.2 and 1.0 mg/mL) were solubilized in a mobile phase (0.025 mol/L sodium phosphate buffer pH 7.4 and 0.15 mol/L NaCl) and sonicated for 10 min. Both the mobile phase and samples/standards were filtered through a hydrophilic polytetrafluoroethylene membrane (PTFE; 0.45 µm). The sample injection volume was 100µL and the running time equal to 65 min. Standards with different molecular weights (MW) were used, such as: α-lactoalbumin (14178 Da), Insulin (5807.6 Da), Vitamin B12 (1355.4 Da) and L-β-4-Dihydroxyphenylanine (197, 2 Da) for the construction of the analytical curve (log MW x TR) and calculation of the percentage of MW distribution by retention time range (TR).

2.12. Hydrophobicity profile

The YPC, GPH and GIPH hydrophilicity profile was determined by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Shimadzu HPLC system, with photodiode array detector (PDA) (Shimadzu, Japan), C18 Luna 100 Å column (4.6 mm x 250 mm; 5 μm particle) (Phenomenex, CA, USA). The composition of the solvents was: solvent A- Milli-Q water with 0.1% trifluoroacetic acid (TFA); solvent B– acetonitrile with 0.1% TFA, filtered on a hydrophilic polytetrafluoroethylene membrane (PTFE; 0.45 μm). The column was maintained at room temperature, with a flow of 1 mL/min, with detection at 214 nm, volume of 50 μL injection, and running time of 55 min. The samples (3 mg protein/mL for hydrolysates and 1 mg/mL for intact YPC) were eluted in a linear gradient of 5 to 20% of solvent B in 20 min, reaching 40% of solvent B in another 20 min and up to 80% in the following 10 min. In the last 5 min of running, the condition was returned to 5% of solvent B.

2.13. nanoLC-ESI-MS/MS

Hydrolyzates GPH and GIPH were resuspended in water:acetonitrile:formic acid (97.9:2:0.1, v:v:v) and subjected to analysis by nanoflux liquid chromatography coupled to sequential mass spectrometry with Electrospray Ionization (nanoLC-ESI-MS/MS), performed on a NanoLCDionex Ultimate 3000 system (Thermo Fisher Scientific), coupled to an Impact II quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker Daltonics). The peptides were retained in the Acclaim Pepmap nano-trap column (Dionex-C18, 100 Å, 75 μm x 2 cm) and separated in line using the Acclaim Pepmap RSLC analytical column (Dionex-C18, 100 Å, 75 μm × 15 cm) under gradient elution from 2 to 98% of acetonitrile:trifluoroacetic acid (99.1:0.1, v:v) for 180 min, and flow adjusted to 300 $nL.min^{-1}$. Mass spectra of MS precursors were acquired in positive ion mode and MS/MS products acquired were acquired at 2 Hz in the mass range of 50-3000 *m/z* and the branched collision-induced dissociation energy parameters varied from 7 to 70 eV.

2.14 Bioinformatics analysis

Raw MS/MS data files were imported into the PEAKS Studio 8.5 software (Bioinformatics Solution Inc., Waterloo, Canada) for *de novo* analysis and database searches [22]. *De novo* analysis was performed with a precursor mass tolerance of 07 ppm, fragment mass tolerance of 0.025 Da, no specific enzymatic cleavage and oxidation in Met (+15.99 Da) and Pyro-Glu from Q (-17.03 Da) were used as dynamic modifications. As the *Dioscorea cayennensis* database presented few protein sequences, peptides were *de novo* sequenced with an average local confidence (ALC) ≥ 50% and submitted to the database search using SPIDER tools [23], against the Uniprot KB Dioscorea database (71 Swiis-prot sequences and 2703 TrEMBL sequences, downloaded on May 3, 2018 from http://www.uniprot.org/). The false discovery rates (FDRs) for proteins and peptides were fixed at a maximum of 1%. Peptides with ALC > 90% unmatched in the homology database were considered as complementary analysis.

2.15 Statistical analysis

For statistical analysis, the software program GraphPad version 6.0 was used. The results were expressed as mean ± SD. ANOVA test was used, followed by Student's t test. Statistically significant differences were considered when $p < 0.05$.

3. Results and discussion

3.1. Yam Protein

The percentage of yam protein, protein extract and YPC, as well as the protein extraction yield is shown in Table 1. The tuber had a protein content of 10.0 g/100g of sample, which is within the range of protein found in yams of other species, as in *D. alata* (15.07 to 8.13 g/100g) [24] and *D. bulbífera* (3.55 to 8.64 g/100g) [25]. After extraction and precipitation at the isoelectric point of the proteins, a concentration corresponding to 64.0 g of total protein per 100 g of sample was obtained.

Table 1. Crude protein content (g/100g) and protein yield: yam tuber (YT), yam protein extract (YPE), yam protein concentrate (YPC).

Samples	Protein content g/100g of sample (dry base)	Protein yield (%)
YT	10,0±0,03*	NM
YPE	29,5±0,04*	66,1
YPC	64,0±0,03*	84,4

NM- not measured
*crude protein content was determined by the Kjedhal method with measurement in triplicate on a dry basis.

The process used to concentrate the proteins was able to increase the protein content by approximately 5 times, constituting the major and of most interest component for the research of a proteome. This technique showed a yield of 84.4%, being advantageous regarding other isolation methods, based on the use of ultrafiltration membranes and columns, since the chemical reagents used are of low cost and, in general, the procedure is relatively simple and thus can add economic and nutritional value to an agro-industrial by-product, such as the recovered YPC.

The yam starch is a product identified as "unconventional starch", which has become increasingly attractive due to its potential for application in the development of new products, being added to foods to increase its functionality characteristics. However, after the extraction of this starch and its use as an alternative in the industry, it stands out for generating a co-product that represents a source of functional compounds. The knowledge of the potential of the bioactive compounds of the by-products derived from the extraction of starch, can bring opportunities for their application and add economic value to the production process of yam tubers of the genus Dioscorea. According to Lu et al. [5] dioscorin, the main reserve protein (85%) of tubers of the genus Dioscorea [26, 4], has several biological activities. Therefore, knowledge of the proteomic profile of *D. cayennensis,* as well as its digestibility, has been considered of interest for the evolution of scientific research.

3.2. Two-dimensional electrophoresis- 2DE of YPC

The separation of YPC proteins by 2D SDS-PAGE revealed a number of 55 *spots*, most of them observed with an isoelectric point (IP) in the range between 5.5 and 7.6. There was also a sequence of 13 more intense points (proteins and their isoforms) close to 31kDa, with IP 5.5-9.4. These results are shown in Figure 1.

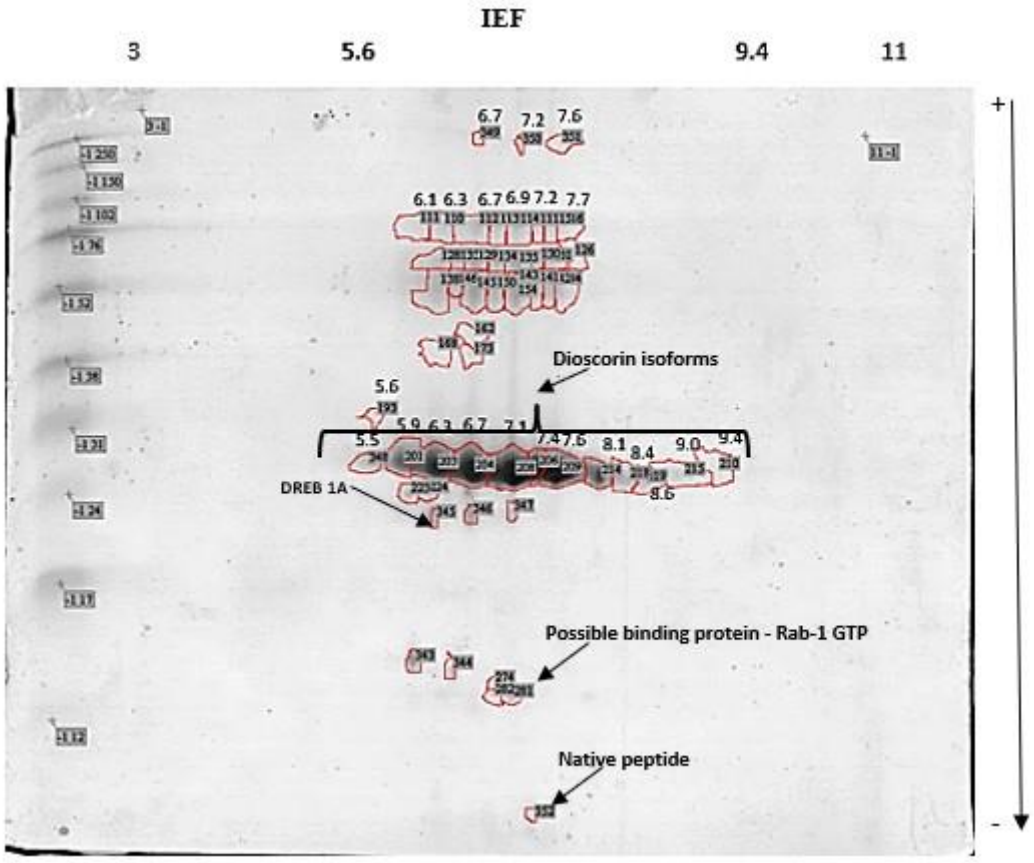


Figure 1. 2D-SDS-PAGE Electrophoretic profile of yam protein concentrate (YPC) pH 3-11 NL-Non-linear.

As shown in Fig.1, the result obtained in this study in 2DE proved to be consistent with other studies carried out on tubers of the same species, which also reported an approximate weight of 31 kDa for the main reserve protein of the genus Dioscorea, dioscorin. Thus, the protein identified with 13 isoforms and most abundant in the 2D gel corresponds to dioscorin, which has been widely studied over the past few years. Initially Conlan et al. [4] identified 6 isoforms of dioscorin (IP 5.68-6.68) in *D. cayenensis*; later the researchers Tsai et al. [27] found 18 isoforms (IP 6.02-6.60) of this same protein for the species *D. pseudojaponica* and 7 (IP 6.60-6.62) for *D. alata*. In a more recent study Sharma, Gupta and Deswal [28] identified 75 spots in tuber *D. alata*, 12 points being identified as isoforms and 27 as precursors of dioscorina, with an approximate weight of 31 kDa.

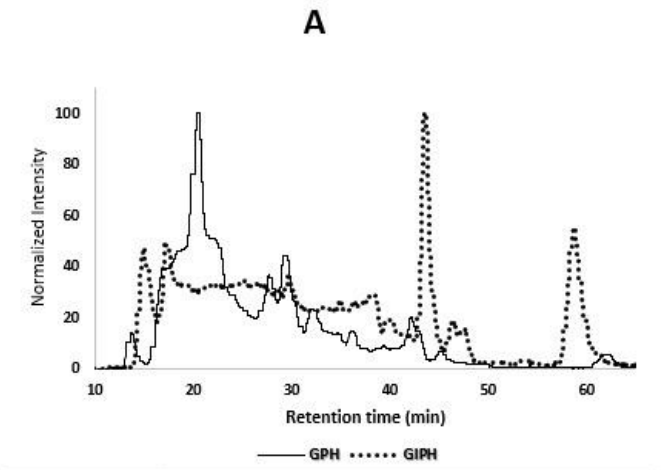
In the protein profile of the YPC, the presence of *spots* with molecular weight equal to or greater than 225 kDa, points close to 102.71 and 56 kDa, as well as between 17 and 12 kDa, were found; the latter may refer to the binding protein Rab-1 GTP with

14.5 kDa, identified in *D. alata*by Sharma, Gupta and Deswal [28]. These authors also verified the presence of DREB 1A with a weight corresponding to 24 kDa. In this research, the presence of a band with 24 kDa, suggests that this protein may also be present in *D. cayenensis.* DREB 1A can play a protective role in tolerating biotic and abiotic stress. According to Bouaziz et al. [29], this class of proteins is responsible for the tolerance of plants during the maturation process. There is also the presence of a molecule with molecular weight <12 kDa, suggesting the presence of native peptide present in the set of proteins identified in the tuber.

3.3. In vitro simulated gastrointestinal digestion of yam proteins

Yam proteins have bioactivity according to Lu et al. [5]. Thereby, further research, through studies of the gastrointestinal protein digest, is important in order to elucidate the protein quality after digestion, as well as the characterization and functionality of the peptides that are produced. Thus, for the knowledge of the tubers protein digestion, the YPC containing 53.7g of protein/100g, was submitted to simulated gastrointestinal digestion (GID) *in vitro,* for the release of peptides in two stages: gastric and intestinal. According to Wang et al. [30], proteins subjected to simulated gastric and intestinal digestion can generate different biological characteristics.

The digestion process includes mechanical, chemical and enzymatic steps to release nutrients and facilitate their absorption. Given the complexity of this biological process, an international consensus on digestion in adulthood has been standardized to simulate this process *in vitro*. For this, a static protocol was elaborated based on physiologically relevant conditions obtained from human beings [11]. The sequence of events that occur during this simulation is close to gastrointestinal digestion *in vivo* [31].



MW range	Molecular weight distribution (%)		Δ between GPH and GIPH (%)
	GPH	GIPH	
> 7 kDa	38.49	26.57	-30.97
5-7 kDa	12.13	6.63	-45.37
3-5 kDa	9.76	10.01	2.60
2-3 kDa	10.74	7.39	-31.19
< 2 kDa	28.88	49.40	71.05

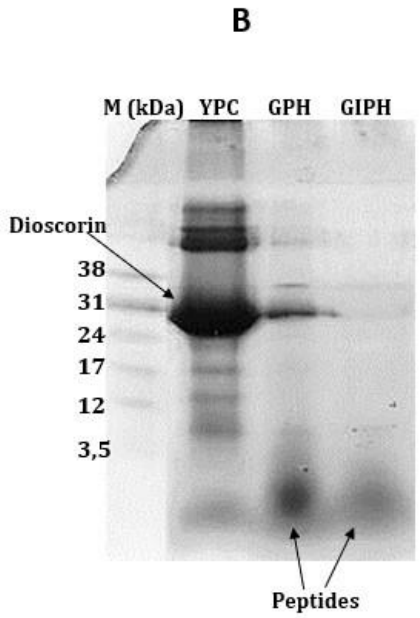


Figure 2. (**A)** Yam protein hydrolyzates molecular weight distribution: size exclusion chromatography – GPH- gastric phase hydrolyzate and GIPH- gastrointestinal phase hydrolyzate. (**B)**: Tricine-SDS-PAGE: M- molecular weight marker; YPC- yam protein concentrate; GPH- gastric phase hydrolyzate; GIPH- gastrointestinal phase hydrolyzate.

The patterns of undigested protein (YPC) and of the digestion phases: gastric (GPH) and intestinal (GIPH) are shown in Fig. 2 (B). The YPC showed protein bands greater than 38 kDa, as well as less than 3.5 kDa. Among which, the most expressive band weighing close to 31 kDa, represents the main storage protein, dioscorin. This result corroborates with studies carried out on tubers of the same species, which also reported an approximate weight of 31 kDa for dioscorin [32,33, 34]. Differences between the GPH and GIPH phases were showed in Figure 2 (A and B). The efficiency of pepsin in the initial hydrolysis of the protein is verified with the molecular mass distribution (Fig. 2A), with approximately 39% of molecules with mass > 7 kDa. In the gel (Fig. 2 B), it appears that even after gastric digestion, the band of the dioscorin protein, although reduced, can still be visualized. This is due to the ability of the pepsin enzyme to nonspecifically digest the macroproteins in polypeptides in the stomach. Hence, in the first phase, the acidic environment of the stomach provided the denaturation of the yam proteins, with consequent unfolding of the molecules and exposure of the peptide bonds, followed by hydrolysis and generation of these polypeptides, for the continued hydrolysis in the intestinal phase of the digestive process [35]. Whereas, polypeptides are digested into smaller fragments only at the intestinal digestion stage [36].

In GIPH, pancreatic enzymes perform most of the proteins digestion in the lumen, in the first portion of the intestine. This process is carried out by intestinal enzymes, which include trypsin, chymotrypsin, elastase and carboxypeptidase, which break the polypeptides into short chains of some amino acids, called oligopeptides, to be subsequently hydrolyzed to dipeptides, tripeptides and free amino acids [35]. With the progression of hydrolysis in GIPH, gastric phase polypeptides are fragmented by pancreatin into oligopeptides. As a result, as shown in Fig. 2 A, larger polypeptides (MW 5-7 kDa) were converted to smaller oligopeptides (MW <3 kDa) after complete GID. In accordance with this result, it can also be seen in Fig. 2 B, a decrease of higher molecular weight proteins and an increase of diffuse bands of low molecular weight peptides. As a consequence, there is a reduction of proteins and polypeptides and a permanence of peptides below 3.5 kDa.

Finally, the *in vitro* GID process showed that the proteins were fragmented into low molecular weight molecules, according to the MW distribution profile, in which approximately 71% of peptides had MW<2 kDa. Therefore, it is suggested that yam proteins are easy to digest by enzymes of the gastrointestinal system. Furthermore, tubers in general are foods that do not have gluten-forming proteins and are recommended for ingestion by individuals with celiac disease. In addition to the importance of protein fragmentation for the digestion and absorption process, [37] points out that many peptides are inactive within the protein matrix, while hydrolysis occurs, the peptides are released. Thus, their ionizable groups, amino acid sequence and functional groups are exposed, with greater capacity to perform different biological activities.

3.4. Composition of total amino acids

The amino acid profile of a protein is of great importance in assessing its nutritional quality. The composition of the total amino acid profile of intact proteins (YPC) and their final hydrolyzate (GIPH) are shown in Table 2.

Table 2. Composition of amino acids and chemical score of yam protein concentrate (YPC) and gastrointestinal phase hydrolyzate (GIPH).

Amino acids (AA)	Samples YPC		GIPH	
Essential	(g/100g protein)	Score	(g/100g protein)	Score
Lys	5.77±0.06	1.28	4.97±0.06	1.10
Trp	1.06±0.02	1.77	2.01±0.11	3.35
Phe	6.95±0.03		6.76±0.05	2.77*
Tyr	3.93±0.02	2.86*	3.76±0.23	
Met	1.90±0.08	0.94**	2.18±0.10	0.94**
Cys	0.16±0.02		0.11±0,01	
Thr	4.35±0.04	1.89	4.20±0,03	1.70
Leu	7.98±0.03	1.35	7.33±0.09	1.24
Ile	4.23±0.03	1.41	3.98±0.08	1.32
Val	5.16±0.05	1.32	4.80±0.10	1.23
His	0.60±0.02	0.40	2.48±0.04	1.65
Non-essential				
Asp	13.29±0.0	NE	13.80±0.23	NE
Glu	17.09±0.1	NE	17.37±0.10	NE
Ser	5.91±0.03	NE	5.88±0.01	NE
Arg	9.87±0.04	NE	9.39±0.03	NE
Ala	4.56±0.03	NE	4.39±0.09	NE
Pro	4.19±0.02	NE	4.11±0.08	NE
Gly	3.80±0.01	NE	4.47±0.10	NE
AA distribution (%)				
Hydrophobic	36.03		35.56	
Hydrophilic	46.62		48.01	
Neutral	17.35		16.43	

Asp = Aspartic acid, Ala = Alanine, Arg = Arginine, Gln = Glutamine, Gly = Glycine, His = Histidine, Cys = Cysteine, Ile = Isoleucine, Leu = Leucine, Lys = Lysine, Met = Methionine, Phe = Phenylalanine, Pro = Proline, Ser = Serine, Thr = Threonine, Glu = glutamic acid, Tyr = Tyrosine, Val = Valine.
Hydrophobic (Ala, Val, Met, Phe, Leu, Ile, Pro,Trp) Hydrophilic (Arg, Asp, His, Lys, Glu) Neutral (Ser, Gly, Thr,Tyr, Cys)
*Fenilalanina+Tirosina; **Metionina+Cisteine. NE- not estimated. Chemical score performed for adults > 18 years (FAO, 2007).

In order to know the nutritional value of yam proteins, as well as their hydrolyzate, the amino acid content was compared with the FAO/WHO [20] recommendation standard for adults. All essential amino acids reached a chemical score > 1, except for sulfur amino acids (methionine and cysteine), with a chemical score of 0.94. Low content of sulfur amino acids is common for most proteins of plant origin, such as beans [38] and sweet potato varieties [39], as well as for tubers of the same genus, of the species *D. alata* [24]. Nevertheless, these limiting amino acids can be complemented with other protein sources in the diet and thus complemented without compromising the balance between nutrients, in order to maintain nutritional quality.

3.5. Composition of free amino acids

In addition to the amino acid composition, digestibility also plays a significant role in the quality of the protein [40]. Only after hydrolysis catalyzed by gastrointestinal tract (GIT) enzymes, proteins are fragmented into polypeptides, peptides and free amino acids, to be then absorbed by epitelial cells [41]. Therefore, the release of free amino acids represents an important parameter to be evaluated to indicate the bioavailability of a protein. In the *in vitro* GID of yam proteins, the combination of enzymatic hydrolysis with pepsin and the pancreatin enzyme system is shown in Table 3, through the release of free amino acids (FAAs) in the final hydrolyzate (GIPH).

Table 3. Free amino acid profile of YPC and GIPH (*Dioscorea cayennensis*)

Free amino acids (g/100g of protein)	YPC***	GIPH**
Asp	0.01±0.00	0.26±0.00
Glu	0.10±0.00	0.29±0.01
Ser	0.00±0.00	0.47±0.00
Gly	0.01±0.00	0.05±0.02
His	0.05±0.59	0.18±0.00
Arg	0.04±0.00	3.82±0.01
Thr	0.02±0.00	0.13±0.00
Ala	0.02±0.00	0.24±0.01
Pro	0.01±0.40	0.12±0.00
Tyr	0.08±0.00	1.65±0.00
Val	0.01±0.00	0.48±0.00
Met	0.03±0.01	0.32±0.02
Cys	0.10±0.03	0.05±0.00
Ile	0.00±0.00	0.30±0.01
Leu	0.02±0.00	1.88±0.00
Phe	0.43±0.04	2.70±0.00
Lys	0.06±0.00	1.47±0.01
Trp	NM*	NM*
Total	0.99±0.04[a]	14.41±0.01[b]

*not measured **Gastrointestinal phase hydrolyzate. ***Yam protein concentrate. Asp = Aspartic acid, Ala = Alanine, Arg = Arginine, Gln = Glutamine, Gly = Glycine, His = Histidine, Cys= Cysteine, Ile = Isoleucine, Leu = Leucine, Lys = Lysine, Met = Methionine, Phe = Phenylalanine, Pro = Proline, Ser = Serine, Thr = Threonine, Glu = glutamic acid, Tyr = Tyrosine, Val = Valine. Different letters: significant difference p <0,5

The concentration of FAAs after the completion of gastrointestinal digestion shows values of Phe (18.77%), Leu (13.07%) and Tyr (11.38%) is relevant, where the first phase of digestion may have contributed to these results. In the gastric phase, the enzyme pepsin mainly

cleaves the peptide bonds of macromolecules between hydrophobic and aromatic amino acids, such as Phe, Leu and Tyr. Despite generating larger fragments, (polypeptides) they expose at the ends the aromatic amino acids that can be cleaved and released in a next step. The trypsin released in the pancreatic juice in intestinal digestion, in addition to hydrolyzing peptide chains, mainly on the carboxylic side, produces morefreeArg (26.46%) and Lys (10.15%). These values were presumably due to the higher hydrolytic activity of trypsin among the residues of these amino acids, in the intestinal phase [42].

It was observed that the proteolytic activity, catalyzed by GIT enzymes, promoted an increase in the concentration of FAAs. In comparison with the intact protein (YPC), higher values are found for most amino acids after hydrolysis, with a significant impact ($p > 0.05$) on the release of FAAs during simulated GID. Although protein digestion results in peptides of different sizes, di and tri peptides, as well as FAAs at the end of digestion, indicate an increase in protein digestibility, with a consequent increase in absorption, at the end of the digestive process.

There is a considerable increase in the concentration of the essential amino acids Tyr, Leu, Phe and Lys released at the end of digestion, which may indicate their bioaccessibility *in vitro* [8]. Ribeiro et al. [43] point out that some small peptides with bioactive potential are able to resist gastrointestinal digestion and reach the mucosa to be absorbed. Thus, one of the important factors to determine the biological activity of peptides, includes the products of digestion, due to the influence on their absorption through enterocytes and bioavailability in target tissues [44].

3.6. Hydrophobicity/hydrophilicity profile of YPC and digested GPH and GIPH

The hydrophobicity/hydrophilicity profile of the YPC, as well as of the peptides present in the GPH and GIPH was carried out using RP-HPLC (Fig. 3). This determination was made in order to elucidate possible interactions that may occur in the human organism, from the release of peptides during simulated digestion *in vitro*.

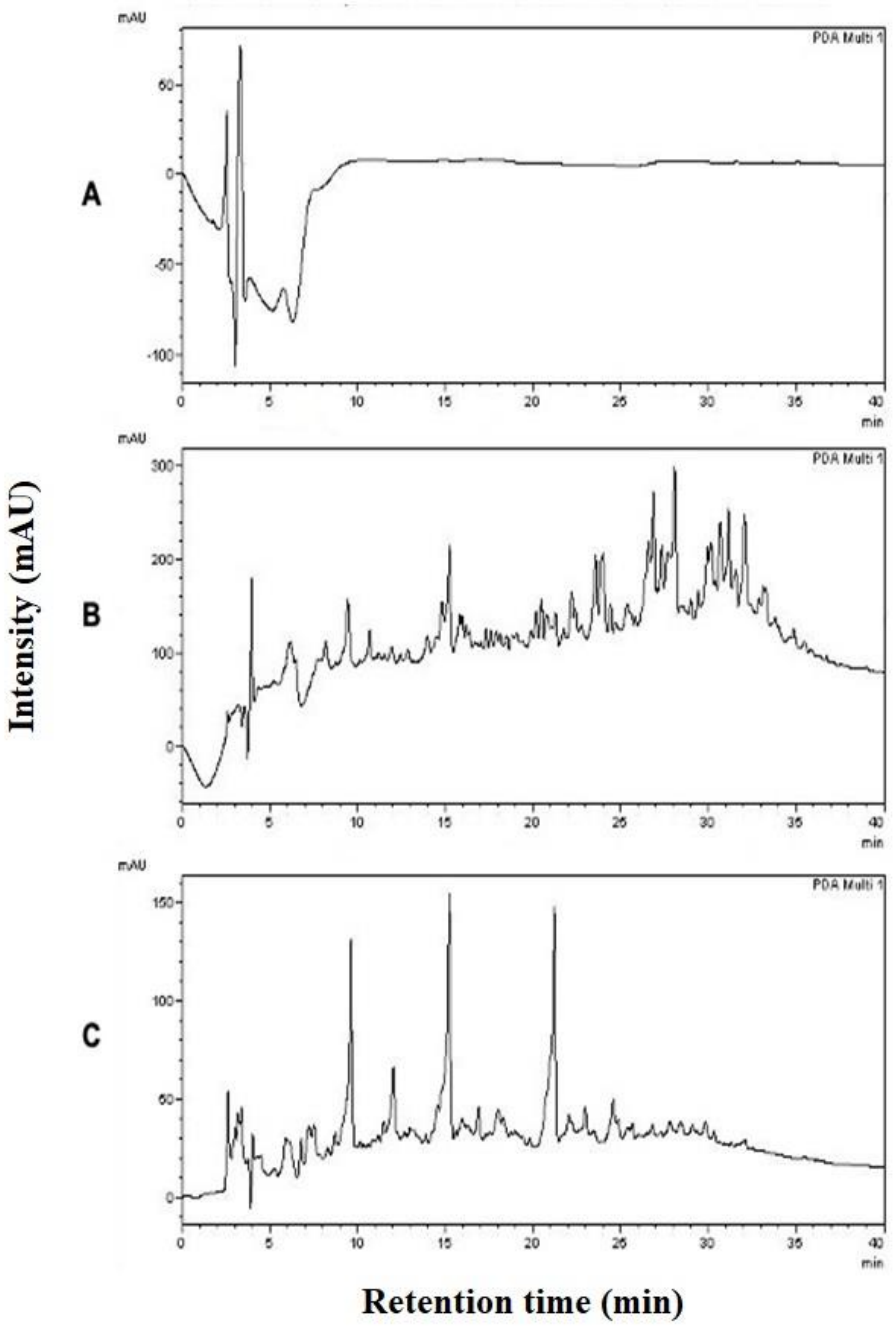


Figure 3. Hydrophobicity profile by RP-HPLC. Samples (A) YPC- yam protein concentrate, (B) GPH- gastric phase hydrolyzate and (C) GIPH- gastrointestinal phase hydrolyzate.

Figure 3 shows the chromatograms obtained from the RP-HPLC undigested protein (YPC) and digested (GPH and GIPH) profiles. In the YPC sample, no peaks were observed during the chromatographic run, revealing that it is insoluble under the conditions of analysis. This is probably due to the fact that the protein concentrated by the isoelectric point has a hydrophobic character, with greater protein-protein interaction and in the absence of interaction

with the solvent of the environment. Thus, the protein was not soluble in the initial mobile phase (ultrapure water with TFA), which resulted in the absence of peaks in the chromatogram referring to the soluble part.

In the GPH digest, there are more significant peaks between 25 and 30 min., whereas the GIPH digest shows peak distribution in retention times close to 10, 15 and 20 min. The chromatograms of protein digestates (Fig 4 B and C) show an increase in solubility regarding YPC. The decrease in peptide peaks at the end of the GIPH, probably occurs due to the result of more intense proteolysis, caused by enzymatic activity and greater release of free amino acids.

The specific bioactivity of peptides of food origin in acting by different mechanisms in the protection of the organism, depends on its structure, physicochemical characteristics, amino acid residues, as well as hydrophobicity/hydrophilicity, and side chain load [45]. As an example, hydrophilic peptides, with repeated proline sequences (IPP, VPP), participate in the mechanism of action of inhibition of the angiotensin-converting enzyme (ACE) and consequent modulation of hypotensive activity [46]. These sequences also show hypoglycemic activity, where they act in the inhibition of dipeptidyl peptidase IV (DPP-IV) [30].

According to Baharand Ren [47] antibacterial and antioxidant peptides have residues of hydrophobic amino acids in their structure capable of scavange DPPH radicals and inhibiting lipid peroxidation. Hydrophobic peptides can also cause the rupture of the negatively charged bacterial membrane [48]. Studies indicate that charge and hydrophobicity are important for the antimicrobial peptides activity [49].

3.7. Identification of peptides from digested GPH and GIPH-nanoLC-ESI-MS/MS

The mass spectrometry analysis performed on the digestion products: GPH and GIPH allowed to identify several peptides that are found within the sequences of the reserve proteins of the genus Dioscorea. Table 4 summarizes peptides released in the GPH and which had reduced molecular weight in the GIPH, as well as those that did not change during digestion.

Table 4. Identification of peptides released in GPH and GIPH related to the sequence of the protein dioscorin of the genus Dioscorea from the database –Uniprot.

Peptide sequences GPH	Mass (Da)	Number of residues	Peptide sequences GIPH	Mass (Da)	Number of residues	Protein source	Number access
NAINNARPLQPTNY	1584.80	14	**NAINNARPLQ**	1109.59	10	*D.cayennensis*	Q39695
KQAVNE**NAINNARPLQPT**NY	2254.14	20	**NAINNARPLQPT**	1307.69	12	*D.cayennensis*	Q39695
KQAVNE**NAINNARPL**QPTN	2091.08	19	**NAINNARPL**	981.53	9	*D.cayennensis*	Q39695
AI**NNARPLQPT**NY	1470.75	13	**INNARPLQPT**	1122.61	10	*D.cayennensis*	Q39695
FSSSQKNEINA**GVVDPN**QLQF	2321.12	21	**GVVDPN**	599.29	6	*D.cayennensis*	Q39695
KNEINAG**VVDPN**QLQF	1784.90	16	**VVDPN**	542.27	5	*D.cayennensis*	Q39695
E**DITWT**	763.33	6	**DITWT**	634.29	5	*Dio 1=D. alata*	A0A1P8PPN9
GRSDPFLSDL	1105.54	10	**GRSDPF**	677.31	5	*D.polystachya*	Q75N35
AINN**ARPLQPL**KF	1480.85	13	**ARPLQPL**	793.48	7	*D.polystachya*	Q75N35
L**SDLEDF**	837.37	7	**SDLEDF**	724.29	6	*D.cayennensis*	Q39695
IKQ**FSSSQ**KNEINAG	1649.83	15	**FSSSQ**	554.23	5	*D.cayennensis*	Q39695
YFEQLK	826.40	6	**YFEQLK**	826.42	6	*D.cayennensis*	Q39695
SINRVAY	949.49	8	**SINRVAY**	821.43	8	*D.cayennensis*	Q39695

GPH- Gastric phase hydrolyzate. **GIPH-** Gastrointestinal phase hydrolyzate

From the set of peptides obtained in the GI digestion and identified by nanoLC-ESI-MS/MS, it was found that the majority, with a convergence factor of 73-78%, belong to the *D. cayennensis* dioscorin protein. The analysis was limited to this protein, because it is the only protein sequence that is deposited in a database [4], since the genome of this species has not been described, so far. The expansion of the search in the database of the genus Dioscorea, also made it possible to identify other peptides within protein sequences by homology with other species of this genus. These proteins include enzymes, such as Glyceraldehyde-3-phosphate dehydrogenase OS (GAPDH2) and Chitinase OS, which participate in the physiological processes of the plant, such as glycolysis and protein expression during tuberization [50]. In addition to the dioscorin isoforms known in the literature, such as *D. alata*: DIO 1 and DIO5; *D. Japonica*: Dj-DIOA1; *D. Pseudojaponi:* Dp-DIOA1 and *D. Polystachya: D1*[24].

On Table 4 it appears that peptides generated in the GPH, such as **KQAVNENAINNARPLQPTNY, GRSDPFLSDL, KNEINAGVVDPNQLQF,** had their molecular mass decreased with the progression of hydrolysis in GIPH, such as: NAINNARPLQ**, GRSDPF, VVDPN,** but maintaining part of the amino acid sequence. These findings indicate that the enzymes (trypsin and chymotrypsin) from the intestine phase were able to cleave the peptide bonds of the larger molecules, with a reduction in their molecular mass at the end of the digestion process. However, other peptides generated in GPH, **YFEQLK** and **SINRVAY**, did not change and remained intact in GIPH, indicating that peptides generated in the stomach can also cross the intestinal barrier without changes.

The beneficial properties of the dioscorin protein [2, 5, 6], may have their bioactive activities potentiated, since many peptides are inactive within the protein matrix, while hydrolysis occurs, the bioactive peptides are released. Therefore, it is evident that the identification of the peptide sequences is essential for the prediction and evaluation of the bioactive role of these molecules in biological processes.

4 Conclusion

The approach of this study was based on the production of *D. cayennensis* yam protein concentrate, as well as its simulated GID *in vitro*, characterization and sequencing of the generated peptides. The results of this study contribute to the knowledge on the profile of

peptides originated from simulated GID *in vitro,* aiming at the prediction of its potential to modulate some biological activities, which would be formed by digestion in the human organism. In this context, they show that the proteins of this tuber are easily digested, with the release of molecules of lower molecular weight, with 71% of peptides with MW< 2 kDa. Thus, the digestion process facilitates the intestinal absorption and utilization for the target organs. However, the peptides obtained after digestion should be explored in future studies, in order to understand the bioactive potential of these molecules for human health.

Conflict of Interest

The authors declare that there is no conflict of interest in this study.

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4.2 ARTIGO II: Submetido ao periódico *Food Chemistry*

Identification of bioactive peptides obtained from simulated *in vitro* gastrointestinal digestion of yam proteins (*Dioscorea cayennensis*)

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Abstract

Bioactive peptides have been widely studied for their contribution to human health. The aim of this study was to identify bioactive peptides generated by simulated *in vitro* gastrointestinal digestion of yam proteins. The yam protein concentrate (YPC) was submitted to simulated gastrointestinal digestion *in vitro*. The fractions after hydrolysis, gastric phase hydrolysate (GPH) and total gastrointestinal phase hydrolysate (GIPH), had their peptides identified by nanoLC-ESI-MS/MS. The identified peptide sequences were subjected to a database-driven (BIOPEP) bioactivity search. *In vitro* bioactivity was assessed by: antioxidative activity (DPPH, ABTS, ORAC), DNA damage protection, antibacterial activity and angiotensin I-converting enzyme (ACE) inhibitory activity. After digestion, the peptides were mostly MW < 3500 Da. A range of peptides with potential sequences for bioactive peptides was identified from the prediction by BIOPEP, such as antihypertensive, anti-diabetic, antioxidant and antibacterial activity. In both GPH and GIPH were found peptide sequences containing proline and leucine (LA**PLPL** LA**PLLP**), which can contribute to the inhibition of ACE, as well as peptides containing aromatic residues in the C-terminal (**D**IT**WT** and **F**LS**WT**) and negative charge in the N- terminal (**DD**C**AY**), which allow the donation of electrons and stabilization of free radicals. These findings suggest that these characteristics contributed to the antioxidant activity in all *in vitro* assays, as well as to the most expressive result in the ORAC assay, with 691 µM of Trolox per mg of sample. Hydrophobic and positively charged peptides (LNQVY**R** and IFDQTLG**KLR**) may have acted in the *Escherichia coli* growth inhibition. The digestion of yam proteins releases potential content of biologically active peptides, which can contribute to the prevention of bacterial infection and chronic degenerative diseases, with beneficial effects to human health.

Keywords: BIOPEP; nanoLC-ESI-MS/MS; Angiotensin I-Converting Enzyme (ACE) Inhibitory Activity; Antibacterial activity; Antioxidative activity.

1. Introduction

In recent years, the investigation of natural bioactive compounds from common foods, either of animal or plant origin, have been intensified aiming to elucidate the benefits of those molecules to human health. The yam of the genus *Dioscorea* is a tuber widely used in African, Brazilian and Oriental cuisine. This tuber has a high carbohydrate content with a low glycemic index, with dioscorin as the major protein, which has been highlighted by the scientific community as a bioactive compound, due to the biological activities shown in *in vitro* and *in vivo* studies (Lu, Chia, Liu and Hou, 2012; Han, Lin, Lin and Hou, 2014).

New approaches have been proposed for the enhancement of the biological activities of food proteins, specially by the release of peptides by *in vitro* simulated digestion (Zhang et a., 2020). *In vitro* digestion tests represent a useful tool to obtain peptides similar to those released in the protein digestion that normally occurs in the human body. Thus, these peptides can be used for predictive studies of biological activities (Lucas-González, Viuda-Martos, Pérez-Alvarez, Fernández -López, 2018). Bioactive peptides derived from foods have been gaining attention due to their potential as biological compounds with high tissue affinity, no side effects, which be used as natural alternatives to replace drugs (Vilcacundo et al., 2018). They can be found encrypted in the protein matrix of food origin (Li-Chan, 2011) and are released after hydrolysis, displaying beneficial effects on the human body (Samardi & Ismail, 2010).

Therefore, recent studies have aimed to elucidate the effects of these low molecular weight compounds, as well as the exposure of amino acid sequences that may be essential for the mechanisms of action and increased bioactivity (Calvo-Lerma et al., 2019). These effects are due to their interaction and participation in different mechanisms of action, which result in physiologically important outcomes such as: inhibition of the angiotensin-converting enzyme (ACE), causing reduction of blood pressure; inhibition of dipeptidyl peptidase-IV (DPP-IV), related to the reduction of blood glucose levels; and antioxidative activity, which reduces the oxidative stress and consequently prevents the onset of degenerative diseases (Li et al., 2020; Zhang et a., 2020; Babini et al., 2017).

The bioactivity of the peptides is related to their chemical structure, composition and sequence of amino acids, which can interact inhibiting the action of enzymes and also stabilizing free radicals, among other interactions (Mojica and Mejia, 2016). Even from different protein sources, peptides with the same key amino acid residues at the active-site are likely to have the same bioactive functions (Vastag, Popovic, Popovic, Krimer-Malesevic and Pericin 2013).

The most recent trend in the prediction of the activity of bioactive peptides involves the use of databases as tools to identify sequences of peptides obtained by simulated *in vitro* digestion. By comparing the generated peptides with bioactive peptides already described in the literature, it is possible to detect new potentially bioactive peptides (Tu, Cheng, Lu and Du, 2018). Thus, the search for similarity between the sequences of new peptides with those of bioactive peptides already described has been proposed as an alternative method of useful application.

Biopep database is one of the bioinformatics tools used to predict or confirm bioactive peptide sequences, which comprises databases of known protein sequences, bioactive peptides and sensory peptides. Therefore, it consists of an integrated program that assists in the prediction of protein hydrolysates (Mooney, Haslam, Holton, Pollastri and Shields, 2013). In addition to this approach, *in vitro* assays help to confirm the bioactive potential of protein molecules generated by hydrolysis and recognized by sequence matching tools. In this context, the present study aimed to identify peptides generated by simulated gastrointestinal digestion of yam proteins and prospect for biological activities using a database-driven search and *in vitro* assays to examine radical scavenging activity, DNA damage protection, antibacterial activity and ACE inhibitory activity.

2. Material and Methods

2.1. Material

Dioscorea cayennensis tubers were collected from the experimental planting fields of the Univesidade Federal of Paraíba, Campus Areia (João Pessoa, PB). Porcine pepsin, porcine pancreatin, bile salts, 2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picryl-hydrazilyl (DPPH), Trolox (6-hydroxy-2,5,7,8-tetramethylchromanic-2-carboxylic acid), Abz-Gly-p-Phe(NO2)-Pro-OH, α-aminobutyric acid and Angiotensin I-Converting Enzime (from rabbit lung) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). [Glu1]-Fibrinopeptide and acetonitrile were purchased from Waters (Milford, MA, USA). All solvents and reagents were of analytical grade.

2.2. Obtention of yam protein concentrate

For protein extraction the yam flour was dispersed in distilled water (1:10, w/v) and the pH was adjusted to 9.0 with NaOH 1 mol.L. The suspension was stirred for 3 h at 25 °C and centrifuged at 3372 × *g* during 30 min at 4 °C. Supernatant was recovered and precipitate was used to repeat the protein extraction process under the same conditions. The supernatants were combined, subjected to protein precipitation with pH adjustment to 5.7 (using HCl 1 mol.L) and centrifuged at 3372 × *g* during 20 min at 4 °C. Supernatant was recovered to repeat the precipitation process, adjusting pH to 4.5 (using HCl 1 $mol.L^{-1}$). The precipitates were combined and neutralized to pH 7.0 with NaOH 1 mol.L. The protein concentrate was dialyzed in Milli-Q water, under magnetic stirring at 25 °C, on a 3.5 kDa MWCO cellulose membrane (Spectrum™ Labs Spectra/Por™, New Brunswick, NJ, USA). Then, the protein concentrate was frozen and freeze-dried (freeze-dryer model LS3000, Terroni Equipamentos, SP, Brazil) to obtain the yam protein concentrate (YPC).

2.3. In vitro *simulation of gastrointestinal digestion and degree of hydrolysis of YPC*

The YPC digestion was performed following the internationally accepted digestion protocol developed by the INFOGEST Cost Action (Minekus et al., 2014). Briefly, 500 mg of YPC were dissolved in 5 mL of deionized H_2O at 37 °C and left in an ultrasonic bath for 30 min. The mixture, previously submitted to pH 2.8 adjustment using HCl 1 mol.L, was added with porcine pepsin (20 mg.mL) and subjected to digestion for 60 min at 37 °C, in a water bath under agitation at 150 rpm (gastric phase). Samples were taken at the starting point and after 60 min of digestion. The gastric phase was interrupted by pH adjustment to 8.0 with NaOH 1 mol.L. The suspension from the gastric phase was mixed with the same volume of simulated intestinal fluid (pH 8.0) containing porcine pancreatin (40 m.mL) and porcine bile extract (24 mg.mL) for 60 min at 37 °C, in a water bath under agitation at 150 rpm (total gastrointestinal phase). The digestion was stopped by heating at 85 ºC for 15 min, followed by centrifugation at 3700 g for 15 min (centrifuge model RC5C, Sorvall Instruments Dupont, Wilmington, USA). Digestions were performed in duplicate.

The hydrolysates of the gastric phase hydrolysate (GPH) and gastrointestinal phase hydrolysate (GIPH) in a 500 Da membrane (Spectrum™ Labs Spectra/Por™, New Brunswick, NJ, USA), recovered, frozen and freeze-dried (Edwards super Modulyo, West Sussex, UK) for further analysis.

The degree of hydrolysis (DH) was determined according to Nielsen, Pertersen, Dambmann (2001), using 6 mM o-phthaldehyde (OPA) (containing 1% SDS, 5.7 mM DTT and

0.17 mol.L disodium tetraborate). The calibration curve was built with serine (0.1 mg.mL). Deionized water was used as blank. DH calculation was performed according to the expression below (Equation 1):

$$DH\ (\%) = \frac{\left[\left(Abs_{sample} - Abs_{blank}\right) \div \left(Abs_{serine} - Abs_{blank}\right)\right] \times \left[(\alpha \times 10) \div (m \times N) - \beta\right]}{h_{tot}} \times 100$$

where α is serine-NH_2 meqv (0.9516), m is the sample mass, N is the nitrogen-to-protein conversion factor (5.75), β and h_{tot} are constants defined for different protein raw materials (soy reference was used, applying 0342 and 7.8 as β and h_{tot}, respectively).

2.4 Matrix-assisted laser desorption/ionization – time-of-fly – mass spectrometry (MALDI-TOF-MS) analysis

MALDI-TOF-MS analysis was performed to molecular mass profiling assessment of peptides obtained from simulated gastrointestinal digestion of Yam. The dried peptide mixture was diluted in ddH2O/ trifluoroacetic acid (TFA) 0.1%, filtered in 0.22 µm-nylon filter and 1uL of peptide solution was mixed (1:1) with a solution of α-cyano-4-hydroxycinnamic acid (α-CHCA matrix, 25 mg/mL) in 0.1% v/v TFA, 30% v/v acetonitrile; 1 µL of peptide mixture in the matrix was placed to an MSP 96 ground steel sample target (Bruker Daltonik, Bremen, Germany) and dried at room temperature. Four spots were prepared for analysis.

MALDI-TOF-MS spectra were acquired with Microflex LT linear mass spectrometer (Bruker Daltonics), using FlexControl software package (version 3.4, Bruker Daltonics). The spectra were recorded in the positive linear mode (laser frequency, 1000 Hz; ion source 1 voltage, 20.05 kV; ion source 2 voltage, 18.35 kV; lens voltage, 6.22 kV; sample rate, 0.50 GS/s; pulsed ion extraction, 230 ns; gain factor, 15.1x) and five independent spectra (1000 shots at random positions on the same target place, for spectrum) were manually collected. Measurements were performed in independent analysis on mass ranges 500–1520 Da, 1500-2600 Da, 2500-3600 Da calibrated externally using Peptide Calibration Standard 2 (Bruker Daltonics) according to mass range specifications.

FlexAnalysis (version 3.4) software packages (Bruker Daltonics) was used for the analysis of all MALDI-TOF-MS data, which included spectral mass adjustment, optional smoothing (using the Savitsky−Golay algorithm with a frame size of 25 Da), spectral baseline subtraction, normalization, internal peak alignment, and peak picking. Pretreated data were then subjected to visualization and statistical analysis.

2.5. NanoLC-ESI-MS/MS analysis of yam hydrolysate fractions

Hydrolysates GPH and GIPH were resuspended in water:acetonitrile:formic acid (97.9:2:0.1, v:v:v) and subjected to analysis by nanoflux liquid chromatography coupled to sequential mass spectrometry with Electrospray Ionization (nanoLC-ESI-MS/MS), performed on a NanoLC Dionex Ultimate 3000 system (Thermo Fisher Scientific), coupled to an Impact II quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker Daltonics). The peptides were retained in the Acclaim Pepmap nano-trap column (Dionex-C18, 100 Å, 75 μm x 2 cm) and separated in line using the Acclaim Pepmap RSLC analytical column (Dionex-C18, 100 Å, 75 μm × 15 cm) under gradient elution from 2 to 98% of acetonitrile:trifluoroacetic acid (99.1:0.1, v:v) for 180 min, and flow adjusted to 300 $nL.min^{-1}$. Mass spectra of MS precursors were acquired in positive ion mode and MS/MS products acquired were acquired at 2 Hz in the mass range of 50-3000 *m/z* and the branched collision-induced dissociation energy parameters varied from 7 to 70 eV.

2.6 Bioinformatics analysis

Raw MS/MS data files were imported into the PEAKS Studio 8.5 software (Bioinformatics Solution Inc., Waterloo, Canada) for *de novo* analysis and database searches (Zhang et al., 2012). *De novo* analysis was performed with a precursor mass tolerance of 07 ppm, fragment mass tolerance of 0.025 Da, no specific enzymatic cleavage and oxidation in Met (+15.99 Da) and Pyro-Glu from Q (-17.03 Da) were used as dynamic modifications. As the *Dioscorea cayennensis* database presented few protein sequences, peptides were *de novo* sequenced with an average local confidence (ALC) ≥ 50% and submitted to the database search using SPIDER tools (Han et al., 2005), against the Uniprot KB Dioscorea database (71 Swiis-prot sequences and 2703 TrEMBL sequences, downloaded on May 3, 2018 from http://www.uniprot.org/). The false discovery rates (FDRs) for proteins and peptides were fixed

at a maximum of 1%. Peptides with ALC > 90% unmatched in the homology database were considered as complementary analysis.

2.7 Prediction of bioactives peptides

Occurrence of biological activities was predicted by analyzing the peptide sequences using BIOPEP database (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008). The PeptideRanker (Bioware.ucd.ie) was used to rank the predicted sequences according to its potential ACE-inhibition.

2.8 DPPH radical scavenging activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of the hydrolysates was estimated according to Picot et al. (2010), with slight modifications. Aliquots of 30 µL of YPC, GPH and GIPH samples, previously diluted in distilled water (10-20 mg.mL), were mixed with 1.5 mL of DPPH solution (60 µmol.L^{-1}) diluted in methanol. The mixture was stirred for 60 min, at 25 °C, protected from light. Then, the samples were centrifuged at 14,500 x g for 5 min and the absorbance of the supernatant was measured at 517 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 500-2000 µmol.L, was used as standard. The DPPH scavenging activity (%SA) was calculated as demonstrated by the Equation 2:

$$\% \text{ Scavenging activity (SA)} = [(\text{Blank abs} - \text{Sample abs}) / \text{Blank abs}] \times 100 \quad \text{(Eq.2)}$$

Furthermore, the Trolox equivalent antioxidant capacity (TEAC) was calculated dividing the Trolox concentration from the curve by the final sample concentration in mg.mL.

2.9 ABTS radical scavenging activity

The ABTS [2,2'-azinobis- (3-ethylbenzothiazoline)-6-sulfonic acid] assay was based on the method described by Wiriyaphan; Chitsomboon; Yongsawadigul (2012). Stock solution of ABTS•+ was prepared mixing ABTS•+ (7 µmol.L) and potassium persulfate (140 µmol.L^{-1}), protected from light for 16 h. Fresh working solution was prepared by diluting the stock solution in ethanol to achieve an absorbance of 0.7 ± 0.05 at 734 nm. Trolox (6-hydroxy-2,5,7,8-

tetramethylchroman-2-carboxylic acid), up to 1000 μmol.L, was used as standard. A 20 μL aliquot of each sample (YPC, GPH and GIPH, 10-20 mg.mL) was mixed with 3.0 mL of the ABTS•+ radical solution in test tubes and homogenized on a tube shaker. After 6 min, absorbance was measured at 734 nm. The scavenging of the ABTS•+ radical was calculated according to Equation 3:

% Scavenging activity (SA) = [(Blank abs - Sample abs) / Blank abs] × 100 (Eq.3)

Trolox equivalent antioxidant capacity (TEAC) was calculated as previously described in section 2.9.

2.10 Oxygen radical absorbance capacity (ORAC)

The antioxidant capacity was also determined by using the ORAC method, as described by Huang et al. (2002). Briefly, the reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4). The final assay mixture (200 μL) contained fluorescein (150 μL, 55 nM), radical initiator 2,2′-azobis(2-amidinopropane) dihydrochloride-AAPH (25 μl and 25 μL of antioxidant Trolox (0.05–4.0 μM) or sample (YPC, GPH or GIPH). Fluorescence was recorded during 50 min, every 60 sec, in an Enspire 2300 plate reader (Perkin Elmer, EUA) with 485 nm excitation and 520 nm emission filters. Phosphate buffer (pH 7.4) was used as negative control. Final ORAC value was expressed as μmol Trolox equivalents per g or mg of sample, according to the area under the curve (AUC).

2.11 Protection of DNA damage

DNA damage protection capacity of YPC, GPH and GIPH was performed according to Huang et al. (2010), with slight modifications. The hydroxyl radical (• OH) was generated by the Fenton reaction according to the method of Kohno et al. (1991). The reaction mixture of 15 μL containing YPC or its hydrolysates (10 and 15 mg.mL, respectively), 5 μL of plasmid DNA from *Escherichia coli* (1 mg.mL), 2 μL of 18 mM $FeSO_4$ and 3 μL of 60 M H_2O_2 was incubated at 37 ° C for 30 min. Then, 2 μL of 1 mM EDTA was added to stop the reaction. The blank was performed with only plasmid DNA and the positive control test was performed with all components of the reaction and water. The mixture was then electrophoresed on 1% agarose gel. To visualize the DNA bands, the gel was stained with Gel-Red ™ (Biotium, Inc., Hayward, USA) and photographed by a Transluminator L.Pix Loccus-Molecular Imaging.

2.12 ACE inhibition assay

In vitro inhibition of ACE was assayed as follow (Farias et al., 2006): the hydrolysis of 10 µM Abz-FRK(Dnp)P-OH was continuously measured at 37 ºC in a Shimadzu RF-1501 Spectrofluorophotometer System adjusted to 320 nm (excitation) and 420 nm (emission). The tests were carried out in 0.1 mol.L Tris-HCl buffer pH 7.0 containing 50 mM NaCl and 10 µM $ZnCl_2$. Samples (YPC, GPH or GIPH) were pre-incubated with the enzyme for 1 min before tests begun. ACE inhibition Activity was calculated from two independent assays and expressed as IC50, which corresponds to the concentration of yam concentrate or hydrolysate capable of inducing 50% inhibition of ACE activity. It was determined using the equation $y = 100\%/1+(x/IC50)^S$, where S is a slope factor. The equation assumes that y decreases with increasing x. The IC50 values for the ACE inhibitor were calculated with the software GraFit (Erithacus Software, West susex, UK) (Leaterbarrow, 2001).

2.13 Antibacterial activity

Antibacterial activity against *Escherischia coli*, *Salmonella* sp and *Lysteria monocytogenes* was tested with YPC, GPH and GIPH according to the broth microdilution protocol (M7-A6) from the National Committee for Clinical Laboratory Standards (2003). Each strain, stored at 4 °C, was placed to grow in Brain Heart Infusion (BHI) medium at 35 °C until approximately 1.5×10^8 CFU.mL, with a McFarland scale of 0.5. Microdilution was performed in 96-well flat-bottom microplates with 90 µL of medium per well. Serial dilutions were made in triplicate, with 1 mg.mL of each sample. Then, 10 µL of bacterial suspension (10^6 CFU.mL) was added. Negative control was prepared with medium and the positive control, bacteria and the medium (with no sample). The microplate was incubated at 35 °C in a Multiskan GO spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and bacterial growth was monitored by measuring the absorbance at 625 nm, every 60 min, for 24 h. Results are presented as minimum inhibitory concentration (MIC).

2.10 Statistical analysis

For statistical analysis, the software program GraphPad Prism version 6.0 (GraphPad Software, CA, USA) was used. The results were expressed as mean and standard deviation

(SD). ANOVA test was used, followed by Student's t test. Statistically significant differences were considered when $p < 0.05$.

3. Results and discussion

3.1. Peptidic profiles of yam hydrolysates

The simulated digestion of YPC resulted in degree of hydrolysis (DH) of 29.74% in the GPH and 30.4% in the GIPH. The results suggest that in the first stage of protein digestion, gastric pepsin was quite effective in the ability to hydrolyze a large number of peptide bonds. This can be attributed to the fact that pepsin lacks specificity, causing a substantial breakdown of low molecular weight (MW) proteins in the gastric phase of digestion (Zhang et al., 2010). Molecular weight profile of peptides in the total gastrointestinal digestion is shown in Figure 1.

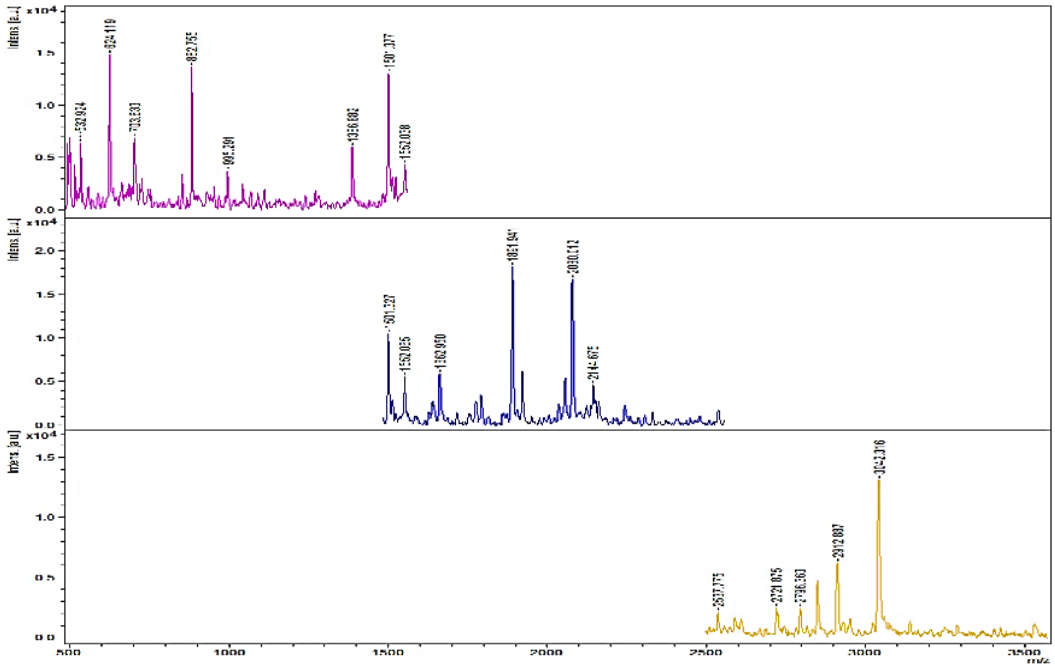

Figure 1. Molecular mass profile of peptides generated in simulated gastrointestinal digestion of YPC-MALDI-Tof

Protein digestion, catalyzed by gastric and intestinal enzymes, has the ability to produce a mixture of peptides of different MW. According to Fig.1, GIPH peptides have MW ranging from 500 to 3500 Da, most of them below 1500 Da. This MW profile is similar to most of the bioactive peptides identified and described in databases (Piovesana et al. 2015). Peptides of low

MW have the ability to interact with various cellular receptors, triggering distinct responses that result in biological activities. For example, Zhang et al. (2020) isolated peptides with MM <3000 Da identified as antioxidant compounds. Indeed, most bioactive peptides have <10 amino acids in their structure (Panchaud, Affolter and Kussmann, 2012).

3.2 Peptide identification and in silico *bioactive potential prediction*

Five hundred and eight peptides were identified in the gastric hydrolysate (GPH), obtained from the gastric phase digestion. Total gastrointestinal simulated digestion resulted in 493 peptides, identified by NanoLC-ESI-MS/MS analysis.

The sequences of the peptides generated by digestion of the YPC were analyzed by computational studies, involving the comparison of the sequences with known bioactive peptides from BIOPEP database. Potential bioactive peptides identified from the sequences found in GPH and total gastrointestinal hydrolysates GIPH, their MW (Da), homologous bioactive sequence and its original source, identification number and protein precursor can be seen in Table 1. Yam peptides showed homology with several bioactive peptide sequences from BIOPEP, whose activities include: ACE inhibition, DDP-IV enzyme inhibition, antibacterial and antioxidant effects.

Table 1. Prediction of bioactive peptides generated after in vitro simulated gastrointestinal digestion. Candidates for bioactive peptides were previously identified based on the Biopep database.

Activity Biological	Sequence Proposal	Mass (Da)	Length	Protein Source	Peptide Sequence Bioactive	ID (BIOPEP)	Precursor protein (BIOPEP)
Gastric phase hydrolysate – GPH							
Antioxidant activity	**DD**C**AY**	586,18	5	*D. cayennensis*	**DD**LPR	8429	*Olea europaea*
	LSINRV**AY**	934,52	8	*D. cayennensis*	**AY**	7866	Residue (Okara)
	SINRV**AY**	821,43	7	*D. cayennensis*	**YAY**	7953	Synthetic peptide
	ELD**FF**	670,31	5	-	TRTGDP**FF**	8755	Rice
	KML**FF**	685,37	5	-	V**PW**	8188	Buckwheat
	TFH**PW**	687,32	5	-	VF**PW**	8189	Buckwheat
					TFE	8220	Synthetic peptide
	LED**PW**	659,30	5	-	VF**PW**	8220	Synthetic peptide
	WGG**PW**	602,27	5	-	**WG,** VF**PW**	9082, 8220	Synthetic peptide
	LLTW	532,31	4	-	**LL**PHH	3772	*Glycine max*
	EDI**TW**	662,29	5	*D. alata*	G**TW**	9165	Synthetic peptide
	TTFFQ	643,30	5	*De novo*	**TT**YY	7871	*Glycine max*
ACE inhibitor **	NAINNA**RP**	868,45	8	-	YANPAVV**RP**	3404	-
	VMAG**GPP**S	714,34	8	*D. cayennensis*	**GPP**	7820	Wheat of gliadin
	LA**PLP**L	622,41	6	*De novo*	**PLP**	2664	-
	LA**PL**LP	622,41	6	*De novo*			
	NNARL**PL**	796,46	7	*D. cayennensis*	**PL**	7513	Fish skin Alaska
	NAINNAR**PL**	981,54	9	-			
	A**VV**SIL	600,38	6	*De novo*	PA**VV**LP	2621	-
	F**VV**DPN	690,00	6	*De novo*	I**VV**E	3260	Synthetic peptide
	VVDGGP	543,00	6	*De novo*	**VVPP**A	7664	-
DPP-IV inhibitor *	LA**PLP**L	622,41	6	*D. cayennensis*	L**PLPL**	8618	Synthetic peptide
	LSDS**PL**	631,33	6	*De novo*	PQNIP**PL**	9058	Synthetic peptide
	LSSYAVTL**PL**	932,30	10	*De novo*			
	PNGPENW	812,37	7	*D. cayennensis*	**PN**	8860	Synthetic peptide

Antimicrobial activity							
	LNQV**YR**	396,72	6	*De novo*	TSK**YR**	8192	-
	QLVHESQDQ**KR**	1366,69	11	*D. cayennensis*			-
	AQLVHESQDQ**KR**	1437,73	12	*D. cayennensis*	KKFHIR**KR**	3684	-
	QQ**LK**	499,28	4	*De novo*	K**LK**LLLLLK**LK**	3648	-
					LKKKKK**LK**KKL**LK**L	3652	-
Gastrointestinal phase hydrolysate – GIPH							
Antioxidant activity	**FL**S**WT**	652,32	5	*Dioscorea polystachy*	**FL**PE	8998	Synthetic peptide
	DIT**WT**	634,29	5	-	P**WT**	8050	-
					R**WT**	8079	-
					L**WT**	8018	-
	FD**EL**	522,23	4	-	**YF**YP**EL**	7887	Casein
	YFQ**ELK**	826,42	6	-	**LK**	8217	-
					L**ELK**	8724	*Phaseolus vulgaris*
	LGTVS**PK**	700,41	7	*D. polystachy*	**LG**FEYY	8240	-
	PFCS**PK**	677,32	6		WEG**PK**	8986	-
					DWD**PK**	9335	-
	VAGLG**DW**	716,34	7	-	KLS**DW**	9222	-
	SW**PWQ**	702,31	5	-	**PWQ**	8047	-
	LRE**PW**	699,37	5	-	**PW**	8190	Buckwheat
	WGG**PW**	601,26	5		VF**PW**	8188	Buckwheat
					WG	9082	
	GIRNPEEI**PW**	805,81	10	*D. oppositifolia*	V**PW**	8189	Buckwheat
	TVF**FY**	676,33	5		Y**FY**	7963	-
					TVYQ	8483	Casein
	TWGG**HQ**	684,29	6	*De novo*	**D**H**HQ**	8135	Marine Bivalve
	DYLELQ	739,36	6	*De novo*	**TW**	8459	Rice (*Oryza sativa* L.)

DPP-IV inhibitor *	DYLE**LQ**	779,37	6	-	**KL**LLRR**LQ**	8716	*Phaseulus vulgaris*
	SDLEDFI**RQ**	876,52	9	-	RLLLKL**RQ**	8732	*Phaseulus vulgaris*
	LREPW	656,80	6	-	**LRE**NN**KL**MLLELK	8726	*Phaseulus vulgaris*
					VIA**PW**	9517	-
	KLGTVSPKQ**VL**	968,90	10	-	**VL**GP	8593	-
	LAPLPL	622,40	6	-	**LPLPL**	8820	-
					LPL	8618	*Phaseulus vulgaris*
					LAPPG	8616	-
					L**LAP**	8788	-
	L**RP**EW	350,69	5	*D. alata*	M**RP**VDPNIE	8689	-
	M**VP**AML	660,33	6	-	W**VP**VDPNIE	8550	-
	E**FPAVP**	658,33	6	-	I**AVP**TGVA	8649	-
	YE**VP**R	662,33	5	-	**VP**	8579	-
	TTT**VDPN**	746,33	7	*D. cayennensis*	M**FPVDP**NIE	8581	-
	V**VDPN**	543,27	5	*D. cayennensis*	M**FPVDP**NIE	9023	-
	GV**VDPN**	600,29	6	-	FRAEH**PL**	8562	Synthetic peptide
ACE inhibitor **	AR**PL**Q**PL**	796,45	8	-	G**PL**	8670	-
	INNAR**PL**	687,50	7	De novo	LHL**PLPL**	7506	-
	LA**PLLP**	692,60	6	De novo	PAVV**LP**	7565	-
	LA**PLPL**	594,70	6	De novo	PQEV**LP**	7586	-
	DALNNAR**LP**	843,56	8	-	ENLHL**PLP**	7765	-
Antimicrobial activity	QVLM**LK**	730,44	6	*D. polystachya*	K**LK**LL**LLLKLK**	3648	-
	Q**LL**QFDDPSY**YR**	1543,73	12	*D. cayennensis*	TSK**YR**	3684	-
	V**LLLK**	1159,59	6	*D. cayennensis*	**LKKKKKLKKKLLKL**	3652	-
	FYFEQ**LK**	695,45	7	*D. cayennensis*	**KK**FHIRK**R**	8192	-
	YFEQ**LK**	973,49	6	*D. cayennensis*			
	QV**LLLK**	826,42	6	*D. cayennensis*			
	KALEDF**LKK**	712,48	9	*De novo*			
	YFQE**LK**	1090,63	6	*De novo*			
	KALEDF**LKK**	826,42	9	*D. cayennensis*			

*DPP-IV dipeptidyl peptidase-IV

** ACE- Angiotensin-converting enzyme

In the first stage of digestion, potential antioxidant peptides were identified (Table 1), such as those containing Y (Tyr), W (Trp) or F (Phe) in C-terminal position: DDC**AY**, SINRV**AY**, EDI**TW**, LLT**W**, KML**FF** and ELD**FF**. Antioxidant peptides from soybeans, wheat, rice and olive proteins, with these amino acid residues in the same position, have already been identified, studied and recorded in the BIOPEP database. According to Elias, Kellerby and Decker (2008), the phenolic structures of aromatic amino acids are highly reactive and participate in the donation of hydrogen, being able to stabilize free radicals such as ABTS.

The peptides generated by GIPH presented potential as antihypertensive compounds, once they showed similarities with the sequence of ACE inhibitors (Table 1). Peptides capable of inhibiting ACE are a key target for the treatment of hypertension, since the angiotensin I-converting enzyme controls blood pressure. According to Nongonierma and FitzGerald (2015), the presence of P (Pro) residues in peptide structure is essential, as it promotes a conformation related to hypotensive effects linked to ACE inhibition. LA**PLPL** for example, a GIPH peptide, presents close similarity to the LHL**PLPL** peptide, a well-known milk ACE inhibitor. Other BIOPEP sequences of analogous peptides have also been identified from wheat and fish proteins as well as synthetic peptides.

After simulated digestion of yam proteins, several inhibitors of the enzyme dipetidyl-peptidase IV (DPP-IV) were also identified, mainly after the final phase, in GIPH. DPP-IV is an enzyme known to inactivate glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). These two gut hormones play an important role in regulating glucose, stimulating pancreatic glucose-dependent insulin and suppressing the release of glucagon. In this way, they promote β cell proliferation and survival, delay gastric emptying and modulate appetite (Lim & Brubaker, 2006). Thus, DPP-IV inhibiting peptides can be considered as an important approach for the treatment of type 2 diabetes due to its hypoglycemic effect (Zhang et al., 2016; Basilicata et al., 2018). According to Lacroix & Li-Chan (2014), peptides presenting P (Pro) residues up to the third position are more resistant to hydrolysis and, therefore, are believed to be more potent DPP-IV inhibitors. As shown in Table 1, the FVV**DPN** and TTTV**DPN** peptides from TH have proline residues (Pro) and similar sequences to other DDP-IV inhibitory peptides.

Structural homologies between ACE inhibitors and DPP-IV are also reported, due to the presence of P (Pro) residues (Nongonierma & FitzGerald, 2015). Yam peptides such as LREPW and FVVDPN, with residues of W (Trp) F (Phe) in the C-terminal and N-terminal positions, respectively, are expected to be good enzyme inhibitors, considering that the presence of

aromatic amino acids (W-Trp, Y-Tyr and F-Phe) participate in the structure of enzyme inhibitors (Lacroix & Li-Chan, 2012).

Regarding antibacterial activity, some structural and amino acid composition parameters are decisive for the occurrence of this biological activity. Antibacterial peptides generally have positively charged amino acid residues, which interact with the anionic surface of the bacterial membrane (Yeman & Yount, 2003; Lee et al., 2011; Kumar, Kizhakkedathu, & Straus, 2018). Hydrophobic and aromatic amino acids (Ala, Val, Leu, Tyr, Phe and Ile) are also alleged contributors to the antibacterial activity of some peptides (Song et al., 2020). Yam GHP has peptides with cationic and amphipathic sequences, such as V**LLLK**, QVL**LLK**, KALEDF**LKK**, showing similarities with antibiotic peptides of the BIOPEP database, with lysine and leucine sequences. Béven et al (2003), when studying helical cationic and amphipathic models of peptides with antimicrobial activities, verified that simple structures composed of two types of amino acids, leucines and lysines (**KLLKLLLKLLLKLLK**), have high antimicrobial activity.

Therefore, to elucidate the predicted bioactive potential through the BIOPEP database, yam hydrolysates generated during simulated digestion of YPC *in vitro* were tested to confirm their bioactivity. An investigation of antioxidant (ORAC, DPPH, ABTS and protection from DNA damage), ACE inhibition and antibacterial activities was then performed.

3.3 Antioxidative activity

The major yam protein, dioscorin, is recognized by its antioxidant activity *in vitro*, through scavenging of DPPH and hydroxyl radicals (Hou et al. (2001). Liu and Lin (2009) performed the simulated digestion of dioscorin using the enzyme pepsin and found that the DPPH elimination activity of the peptic hydrolysates increased with the hydrolysis time. Antioxidative activity of YPC, GH and TH, are shown in Figure 2.

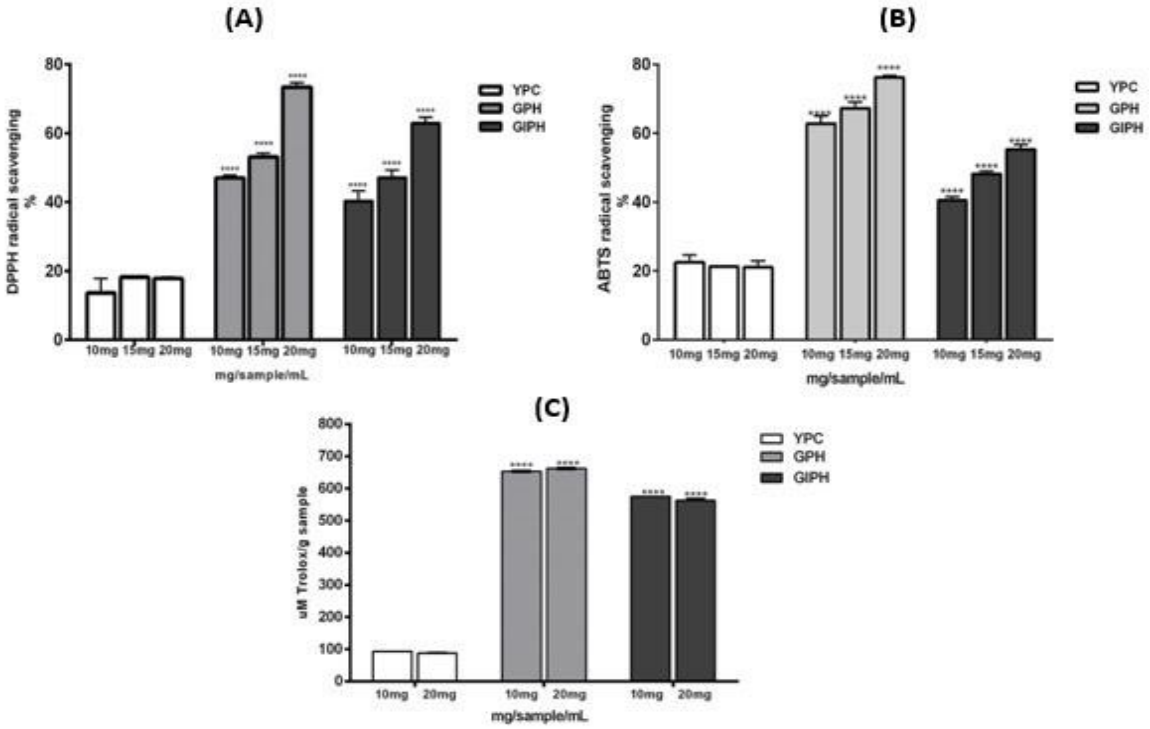

Figure 2. Antioxidant activity of yam protein concentrate (YPC) and simulated in vitro gastrointestinal digestion phases: GPH (gastric phase hydrolysate) and GIPH (gastrointestinal phase hydrolysate), by the methods: (A) scavenging of the radicals DPPH, (B) ABTS and (C) ORAC.

Scavenging activity (SA) of yam hydrolysates was significantly higher than that of intact protein (YPC), confirming that bioactive fragments are released during the progress of digestion hydrolysis. GHP (Figure 2-B) showed SA above 80%, meanwhile SA of GIPH was 60%. Han et al., (2014) also verified antioxidant activity of peptides from *in silico* digestion of dioscorin. The precursor protein structure, as well as the hydrolytic process can affect the bioactivity of the peptides. Bioactive peptides released after hydrolysis processes expose amino acids responsible for the scavenging of free radicals, highlighting that the chemical and structural characteristics of the peptides generated by hydrolysis are important for the antioxidant capacity. Aromatic residues Phe (F) Tyr (Y) and Trp (W) enhance the antioxidant activity, which may occur due to the presence of the phenyl radical, an excellent hydrogen donor for free radicals (Han et al., 2013; Song et al., 2015; Zou et al., 2016; Zhang et al., 2020).

Antioxidative activity may be related to peptide sequences containing tyrosine found in the hydrolysates **(DDCAY** and SINR**VAY**). They were, probably, released from dioscorin, since they have homology with parts of the amino acid sequence of the main yam protein. These peptides also present sequences similar to the antioxidant peptides registered in the BIOPEP

database (Table 1). Amino acid residues at N and C-terminal regions are very important factors for the ability to neutralize free radicals. In this context, Li and Li (2013) studied the relationship between structure and antioxidant activity of peptides with models using quantitative structure activity relationships (QSAR) and found that negative charges at the N1 and N2 positions contributed to results of antioxidant activity, using the ORAC assay. DDCAY peptide in GHP, with residues of Asp (D) in positions N1 and N2, may have contributed to the expressive result for antioxidative activity (Fig. 2 C) in the ORAC assay, with 691 µM of Trolox per mg of sample. Peptides DITWT and FLSWT may also be related to the antioxidative activity, due to their aromatic residue and negative charge at N-terminal, respectively.

Thus, the results suggest that the hydrolysis of yam proteins releases peptides capable of scavenging free radicals. Gastrointestinal digestion produces a plethora of antioxidative peptides which remain intact for intestinal absorption and bioaccessibility and may contribute as exogenous antioxidants to the metabolism

3.4 Protection of DNA oxidative damage

In addition to traditional techniques for assessing the antioxidant capacity of protein molecules, several other procedures, based on electrochemical methods, can be used to evaluate antioxidative activity. Among those, the most important are methods based on protection against oxidation of DNA bases and methods based on the ability to reduce Fe^{3+}. In this study, DNA protection capacity of YPC, GPH and GIPH was also tested, and results are shown in Figure 3.

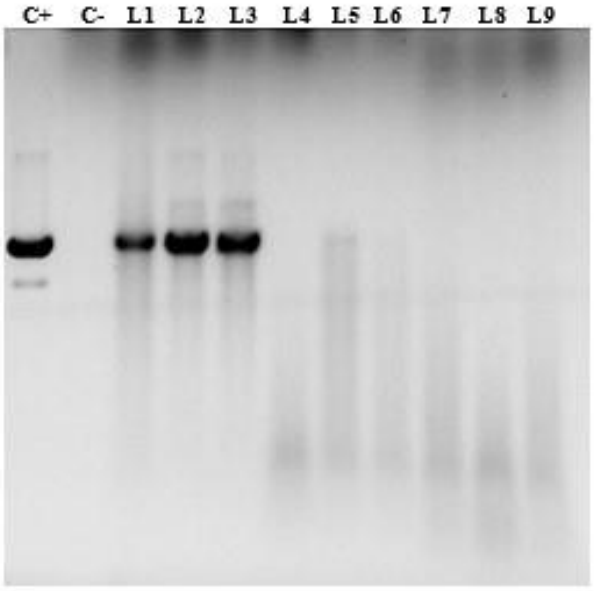

Figure 3. Protective effect of YPC and gastrointestinal digestion phases - GPH and GIPH against oxidative damage to DNA induced by the hydroxyl radical produced from the Fenton reaction between H_2O_2 and iron. C + (plasmid DNA only); C- (plasmid DNA + Fenton's reagent); L1-GIPH 10mg/mL; L2-GIPH 15 mg/mL; L3-GIPH 20 mg/mL; L4-GPH 10 mg/mL; L5-GPH 15mg/mL; L6- GPH- 2 0mg/mL; L7-YPC 10 mg/mL; L8-YPC 15mg/mL; L9- YPC- 20 mg/mL.

DNA bands resulted from tests with samples and negative control can be observed on Fig. 3. The protective activity against DNA damage was shown in lines L1-L3, referring to the different concentrations of GIPH (the sharper the bands, stronger the DNA protection conferred by the sample). On the other hand, for all YPC concentrations there is degradation of the DNA bands, whereas opaque bands in the GHP indicate low protective action or the destruction of the DNA structure, through the reaction of the radicals •OH produced in the Fenton reaction. Free radicals are one of the main causes of DNA damage, being related as causes of the appearance or spread of degenerative diseases (Duracková, 2010).

According to Saiga, Tanabe and Nishimura (2003), peptides presenting Glu and Asp amino acids at the C-terminal contribute to the protection of DNA, inhibiting metal-mediated oxidation processes. Therefore, the peptides **DD**CAY, **D**ITWT and **ED**ITW, obtained by simulated gastrointestinal digestion (Table 1), are apparently involved with the yam hydrolysate ability of eliminating free radicals and protect DNA from damages induced by the •OH radical. Furthermore, GIPH also presents peptides such as **D**IT**WT** and **F**LS**WT**, to which antioxidant activity demonstrated by the ORAC method was linked. Therefore, these results suggest that the protection of DNA molecules against free radicals by GIPH can be effective in protecting biomolecules in the human body.

3.5 ACE inhibition activity

Five of the peptides generated by simulated gastrointestinal digestion and further identified, exhibited a high score for antihypertensive potential. The Peptide Ranker scores, predicted for the peptides most likely to be bioactive, are shown in Table 2. These results were confirmed by *in vitro* tests, whose outcomes strongly suggest that the digestion of yam proteins produces peptides that may be directly linked to the ACE inhibition presented in this study (Fig.4).

Table 2. Peptide Ranker score for bioactivity of peptides obtained from simulated gastrointestinal digestion of YPC predicted for ACE inhibition

Serial number	Peptide sequence	Digestion phase	*Score* Peptide Ranker
1	LAPLPL	GPH/GIPH	0.79
2	LAPLLP	GPH/GIPH	0.76
3	ARPLQPL	GIPH	0.71
4	LREPW	GIPH	0.62
5	NNARLPL	GIPH	0.50

GPH- gastro phase hydrolysate; GIPH- gastrointestinal phase hydrolysate

Peptide Rank is useful for tracking a large set of data for sequences of bioactive peptides, since it classifies them according to their biological potential, through records in databases. Results above a score of 0.5 mean that there is an enhanced probability of a peptide displays a bioactivity. The closer the predicted probability of score 1, the greater the reliability of bioactivity. This experimental screening allows focusing on peptides in a subset, leading to a better understanding of biological activities and also to the synthesis of bioactive peptides (Mooney et al., 2012; Salim and Gan, 2019).

Among the selected peptides with bioactive potential (Table 2), the amino acid Pro (P) is present in all sequences. Four of them have Leu (L) and Pro (P) sequences, such as LA**PLPL**, LA**PLLP**, AR**PL**Q**PL**, NNARL**PL**. ACE inhibition activity is expected to be found for peptides with greater frequency of these two amino acids in their composition, as well as lysine. In fact, ACE inhibitory peptides are characteristically short and carry hydrophobic residues, including proline (Rao et al., 2012; Danish et al. 2017). Previous studies have shown that the peptides Isoleucine-Proline-Proline (IPP) and Leucine-Lysine-Proline (LKP), are ACE inhibitors, therefore, they have the potential to maintain normal blood pressure (Bütikofer, Meyer, Sieber, Walther, & Wechsler, 2008; Fujita & Yoshikawa, 1999). According to Girgih

et al. (2014), ACE inhibition is influenced by the presence of hydrophobic and branched-chain amino acids in the peptide structure, as well as the presence of aromatic amino acids (LREPW) at the C-terminal. The amino acids located in the three positions close to the C-terminal are considered as determinants for competitive binding to the ACE active site, inhibiting the enzyme (Hernández-Ledesma, Contreras and Recio, 2011).

The LA**PLPL** and LA**PLLP** peptides, with a score of 0.79 and 0.76, respectively, were generated in the gastric phase of the digestion, but remained intact in GIPH. Intact peptides, resistant to intestinal enzymes, can reach the brush border membrane and may be transported across it to bloodstream. Furthermore, these peptides are of low molecular weight, which facilitates their access to active sites, such as the ACE inhibition site (Salim and Gan, 2019).

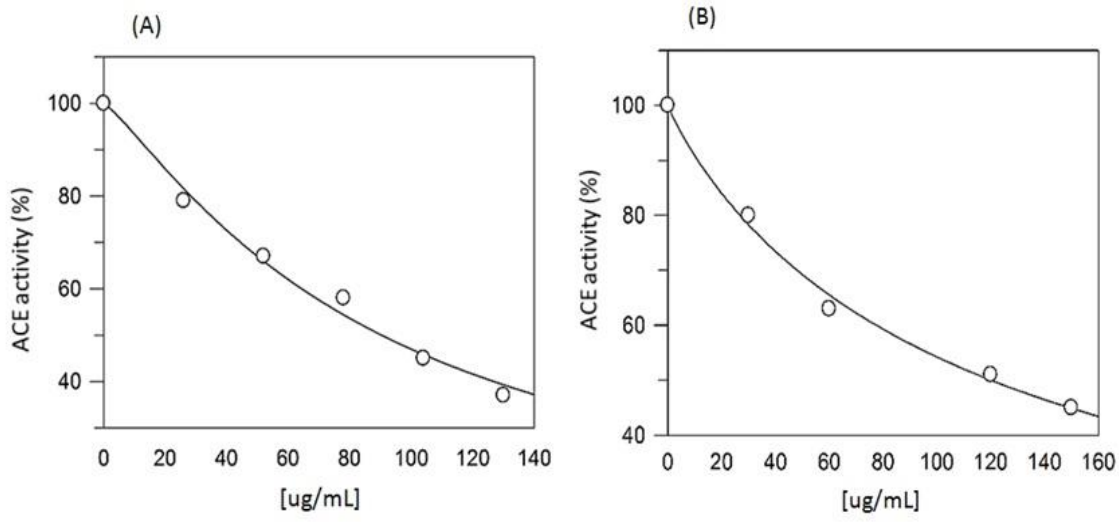

Figure 4. ACE* inhibition ctivity by in vitro simulated gastrointestinal digestion fractions. (A) Gastric phase hydrolysate (GPH) and (B) gastrointestinal phase hydrolysate (GIPH).
*Angiotensin-converting enzyme (ACE).

As shown in Fig. 4, the inhibition of ACE reached IC50 of 90 µg.mL for GPH and 120 µg.mL GIPH. In another study, the action of the IPP peptide on ACE inhibition was evaluated, finding an IC50 of 2,819 µg.mL (Danish et al., 2017). The intact protein (YCP) did not exhibited activity (data not shown), probably due to its low solubility and high molecular weight structure, where the bioactive peptides are encoded inside the molecule. Other authors also reported direct relation between the increase of solubility and the enhancement of antihypertensive effects when proteins are submitted to simulated digestion (Priyanto et al., 2015; Liu et al., 2020). This may occur due to the specificity of trypsin, which cleaves peptide bonds containing lysine and arginine, leading to the exposure of these amino acids (Lin et al.,

2013). Toopcham et al. (2017) found that positively charged amino acids, such as lysine and arginine, have the ability to increase the effectiveness of ACE-inhibiting peptides, due to the high affinity for the ACE active site.

Our findings suggest that *in vitro* gastrointestinal digestion can contribute to the ACE inhibitory activity of yam proteins. This can be promising, since hypotensive effects of a peptide obtained by hydrolysis have also been observed in an *in vivo* model of spontaneously hypertensive rats (Fujita and Yoshikawa, 1999). It is worthy of note that proline-rich pentapeptides have been detected in human plasma after oral administration, which demonstrates that intestinal absorption has occurred in the human body (Vanplaterink et al., 2006).

3.3.4 Antibacterial activity

Simulated digestion produced antimicrobial peptides against *E. coli* (Table 3). Most expressive antibacterial activity ($p < 0.5$) was observed after the total gastrointestinal digestion of yam proteins: GIPH had MIC of 0.094 mg.mL meanwhile GPH and YPC had MIC of 0.188 and 1.5 $mg.mL^{-1}$, respectively. This can be explained by the cleavage of peptide bonds and the consequent decrease of the molecular weight of protein molecules, since smaller molecules can easily penetrate the bacterial cell membranes, creating pores, causing cell leakage and damage to the bacteria (Epand and Vogel, 1999). Moreover, hydrolysis generates a mixture of peptides which expose different electrical charges and polarity. Our findings agree with Hwang et al. (2016), who found the best results for inhibition of Gram-negative bacteria, such as *E. coli*, for linseed peptide fraction with lower molecular weight.

Table 3. MIC* per sample in the growth of *S. aureus, E. coli, L. monocytogenes e Salmonella spp* after 24 h of incubation.

Amostra (MIC/mg/mL)	**Bacteria**		
	Salmonella spp	*Escherichia Coli*	*Listeria monocytogenes*
YPC	NI	1,5 a	NI
GPH	NI	0,188b	NI
GIPH	NI	0,094c	NI

NI-Did not inhibit. *MIC- Minimum inhibitory concentration (MIC). YPC- yam protein concentrate. GPH- gastric phase hydrolysate. GIPH- gastrointestinal phase hydrolysate. Different letters represent a significant difference between the samples p/<0,05

According to Ciumac et al. (2019), to act as antibacterial, peptides need to fulfil some requirements, such as: favorable amino acid composition, amphipathicity, possess electrical charges and reduced molecular weight. Previous studies have also reported that antimicrobial peptides are characterized as cationic and amphipathic (Nielsen et al., 2007). Fractions of cottonseed peptides with a higher number of positively charged amino acids were found to be essential for the inhibition of *E. coli* strains (Song et al., 2020). An antibacterial peptide containing positively charged amino acid (Arg) was also reported by Chan et al. (2006).

It is likely that the GH peptide LNQV**YR** and the TH peptide IFDQTLG**KLR** contributed to the inhibition of the *E. coli*, due to the presence of arginine (R). Furthermore, the peptides QQ**LK**, QVL**LLK** and V**LLLK**, with sequences of aliphatic and cationic amino acids, such as Leu (L) and Lys (K), may also be involved with the antibacterial activity of the yam hydrolysates. The electrical charge allows interaction with the gram-negative bacteria membrane (Lee et al., 2011). Yam concentrate and hydrolysates did not display inhibition activity against *Salmonella* and *L. monocytogenes* growth.

Conclusion

After simulated gastrointestinal digestion of the proteins of yam tubers *D. cayennensis* several peptides with bioactive potential were identified. Using i*n silico* tools in the search for homologous sequences, we found peptides sharing strong similarity with known sequences capable of act as antioxidative, ACE inhibitor, DPP-IV inhibitor and antibacterial. *In vitro* tests were able to confirm the predictive investigation and pointed out that peptides containing proline and leucine can be related to the hypotensive and hypoglycemic activities identified in yam hydrolysates. Peptides containing aromatic residues and negative charge can contribute to the antioxidative activity. Amphipathic peptides, such as leucine and lysine, seems to be responsible for the observed antibacterial activity. These results reinforce that the digestion of yam proteins, including dioscorin, releases fragments of peptides with natural bioactive sequences during the hydrolysis process. Thus, apart from the fact that yam is a rich source of nutrients, our results show that yam proteins are also sources of bioactive peptides, promising molecules which can contribute to human health, either reducing the risk of development of chronic degenerative diseases or preventing/fighting against bacterial infections.

Conflict of Interest

The authors declare that there is no conflict of interest in this study.

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5 CONSIDERAÇÕES FINAIS

O concentrado proteico obtido teve rendimento de aproximadamente 54%, com perfil de aminoácidos totais característico de uma proteína de origem vegetal, apresentando todos os aminoácidos essenciais dentro das recomendações, exceto os sulfurados. Além disso, os resultados obtidos neste estudo indicam que a digestão gastrointestinal simulada *in vitro* foi eficiente para hidrolisar as proteínas do concentrado proteico de inhame, cujos peptídeos obtidos, em grande parte, foram gerados a partir da hidrólise da dioscorina, principal proteína de reserva do inhame.

A partir do CPI foram liberados peptídeos de baixo peso molecular, apresentando teores consideráveis de aminoácidos aromáticos, hidrofóbicos e ácidos com potencial bioativo. Ademais, os peptídeos apresentaram perfil hidrofóbico, característica essencial para atividade antibacteriana e interação com radicais livres. As sequências dos peptídeos gerados por hidrólise apresentaram predição para bioatividades como antioxidante, antidiabetes, inibidor de ECA e antibacteriano quando confrontadas com aquelas de peptídeos bioativos da base de dados BIOPEP®. Em adição, o hidrolisado após digestão gastrointestinal simulada, apresentou atividades biológicas antioxidante, antibacteriana e hipotensora *in vitro*.

Estes resultados indicam que a digestão das proteínas do tubérculo inhame pode liberar moléculas proteicas de baixo peso, que podem participar de diferentes mecanismos de ação no organismo humano, contribuindo para a promoção da saúde. Desta forma, o inhame tem potencial como alimento funcional, devido as propriedades biológicas de suas proteínas. Portanto, este tubérculo deve ter seu consumo estimulado, transpondo assim as barreiras do regionalismo. No entanto, outros estudos são necessários para avaliar os efeitos que a digestão pode produzir *in vivo*, no intuito de constatar a efetividade destas atividades bioativas em humanos.

ANEXOS

ANEXO A- Comprovante de submissão do Artigo I

Dear Dr. Edilza Nascimento,

You have been listed as a Co-Author of the following submission:

Journal: Food Chemistry
Title: Identification of bioactive peptides released from in vitro gastrointestinal digestion of yam proteins (Dioscorea cayennensis)
Corresponding Author: Carlos Gadelha
Co-Authors: Edilza S Nascimento; Katya Anaya, Ph.D.; Julia C Oliveira; José Thalles J Lacerda, Ph.D.; Michael E Miller; Meriellen Dias; Maria Anita Mendes; Juliana A Pallone, Ph.D.; Clarice W Arns, Ph.D.; Maria Aparecida Juliano, Ph.D.; Tatiane S Gadelha, Ph.D.; Maria Teresa B Pacheco, Ph.D.;

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ANEXO B- Comprovante de submissão do Artigo II

Journal: International Journal of Biological Macromolecules

Title: %ARTICLETITLE%

Corresponding Author: %CORRAUTHNAME%

Co-Authors: Edilza Silva do Nascimento; Julia Mariano Caju de Oliveira; José Thalles Jocelino Gomes de Lacerda; Samara Batista Montenegro; Maria Elisa Caetano-Silva; Mariellen Dias; Maria Anita Mendes; Luciano da Silva Pinto; Maria Aparecida Juliano; Tatiane Santi-Gadelha; Maria Teresa Bertoldo Pacheco

Manuscript Number:

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