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Avaliação da bioatividade e identificação de peptídeos bioativos de hidrolisado antioxidante de soja

Evaluation of bioactivity and identification of bioactive peptides from antioxidant soybean hydrolysate

Rio de Janeiro,

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Orientador: Dra. Maria Gabriela Bello Koblitz.

Coorientador: Dr. Anderson Junger Teodoro.

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Dedico este trabalho a Deus, pois ele me sustentou durante todos esses anos, e aos meus pais, que mesmo em momentos difíceis, não mediram esforços na realização dos meus sonhos.

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EPÍGRAFE

“Você nunca sabe a força que tem até que sua única alternativa é ser forte”

RESUMO

A soja é consumida como alimento há milhares de anos e, recentemente, vem tornando-se cada vez mais popular entre os consumidores por preocupações ecológicas, éticas e de saudabilidade. Suas propriedades benéficas à saúde se devem à presença de componentes biologicamente ativos, como isoflavonas, saponinas, inibidores de protease e peptídeos. Resumidamente, sua atividade está relacionada ao seu potencial preventivo contra doenças cardiovasculares, diabetes, sintomas da menopausa, osteoporose, câncer de próstata e mama. Nesse sentido, o objetivo principal deste estudo foi gerar peptídeos com atividade biológica a partir da hidrólise da proteína de soja e então avaliar seu potencial funcional. Para tal, o presente trabalho foi dividido em quatro etapas. Nas duas primeiras etapas, foram realizados levantamentos bibliográficos: sobre a soja e seus peptídeos e sobre peptídeos especificamente com função antidiabética. As pesquisas foram realizadas em bases de dados como PubMed, Scopus, Google Scholar e Scielo, considerando artigos publicados em português, inglês e espanhol entre os anos de 2017 e 2022. Na terceira etapa, o hidrolisado de concentrado proteico de soja foi produzido utilizando papaína, uma protease raramente testada para produção de bioatividade. Os peptídeos foram separados por ultrafiltração (< 3 kDa (LMMH) e > 3 kDa (HMMH)) e sequenciados por meio de LC-MS/MS. Foi então realizada uma avaliação *in silico* desses peptídeos. Para a análise *in vitro* foram avaliadas as atividades antioxidante e inibidora de enzimas digestivas (alfa-glicosidase e lipase). Sessenta e nove peptídeos foram identificados na fração HMMH e 32 na LMMH, mas apenas 16 corresponderam às 118 sequências obtidas por hidrólise simulada *in silico*. A fração HMMH apresentou maior atividade antioxidante, por todos os 5 métodos *in vitro* aplicados (DPPH, ABTS, FRAP, ORAC e TBAR`s). Ambas as frações mostraram atividade inibitória semelhante contra alfa-glicosidase e lipase pancreática, o que torna este o primeiro relato de atividade inibitória de lipase pancreática por peptídeos obtidos de proteínas de soja. Na quarta etapa, esses mesmos peptídeos foram avaliados quanto aos efeitos antiproliferativos e apoptóticos em linhagem celular de câncer de próstata humano (PC-3). A viabilidade celular foi determinada por ensaio de MTT e o ciclo celular e apoptose por citometria de fluxo. Todas as amostras apresentaram atividades citotóxicas contra a linhagem PC-3 em 48h. hidrolisado total (TH) e não hidrolisado (NH) induziram a parada do ciclo celular na fase G0/G1. Além disso, TH e LMMH induziram a apoptose de células PC-3. Assim, os hidrolisados de soja obtidos com papaína se mostraram uma boa fonte de peptídeos antiproliferativos contra o PC-3, enquanto o TH pode ter potencial para ser desenvolvido como suplemento nutricional em uma terapia alternativa de câncer, além de agregar valor a produtos adicionados de soja. Ensaios clínicos em animais e humanos devem ser realizados para melhor compreensão e confirmação dos achados obtidos no presente estudo.

Palavras-chaves: peptídeos bioativos, soja, antioxidante, antidiabéticos, câncer, próstata

ABSTRACT

Soy has been consumed as a food for thousands of years and, recently, it has become increasingly popular among consumers due to ecological, ethical and health concerns. Its beneficial health properties are due to the presence of biologically active components, such as isoflavones, saponins, protease inhibitors and peptides. Briefly, its activity is related to its preventive potential against cardiovascular diseases, diabetes, menopausal symptoms, osteoporosis, prostate and breast cancer. In this sense, the main objective of this study was to generate peptides with biological activity from the hydrolysis of soy protein and then evaluate their functional potential. To this end, the present work was divided into four stages. In the first two stages, bibliographic surveys were carried out: on soybean and its peptides and peptides specifically with an antidiabetic function. The searches were carried out in databases such as PubMed, Scopus, Google Scholar and Scielo, considering articles published in Portuguese, English and Spanish between 2017 and 2022. In the third step, the soy protein concentrate hydrolyzate was produced using papain, a protease rarely tested for bioactivity production. The peptides were separated by ultrafiltration (< and > 3 kDa, LMMH and HMMH, respectively) and sequenced using LC-MS/MS. An *in silico* evaluation of these peptides was then performed. For the *in vitro* analysis, the antioxidant and inhibitory activities of digestive enzymes (alpha-glucosidase and lipase) were evaluated. Sixty-nine peptides were identified in the HMMH fraction and 32 in the LMMH, but only 16 corresponded to the 118 sequences obtained by simulated hydrolysis *in silico*. The HMMH fraction showed higher antioxidant activity, by all 5 *in vitro* methods applied (DPPH, ABTS, FRAP, ORAC and TBAR`s). Both fractions showed similar inhibitory activity against alpha-glucosidase and pancreatic lipase, which makes this the first report of pancreatic lipase inhibitory activity by peptides obtained from soy proteins. In the fourth step, these same peptides were evaluated for antiproliferative and apoptotic effects in human prostate cancer cell lines (PC-3). Cell viability was determined by MTT assay and cell cycle and apoptosis by flow cytometry. All samples showed cytotoxic activities against the PC-3 strain within 48h. TH and NH induced cell cycle arrest in the G0/G1 phase. Furthermore, TH and LMMH induced apoptosis of PC-3 cells. Thus, soy hydrolysates obtained with papain proved to be a good source of antiproliferative peptides against PC-3, while total hydrolysate (TH) may have the potential to be developed as a nutritional supplement in an alternative cancer therapy, in addition to adding value to added soy products. Clinical trials in animals and humans should be performed to better understand and confirm the findings obtained in the present study.

Keywords: bioactive peptides, soybean, antioxidant, antidiabetics, cancer, prostate

SUMÁRIO

O presente trabalho segue as normas da tese no formato de artigo definido pelo Programa de Pós-graduação em Alimentos e Nutrição em 14 de maio de 2019.

Assim esta tese, está dividida em 4 capítulos:

I) Capítulo 1 - Artigo de revisão bibliográfica: "Soja e seus peptídeos – Uma breve revisão". Artigo que traz uma revisão sobre a história da soja, suas proteínas e peptídeos.

II) Capítulo 2 - Artigo de revisão bibliográfica: "Critical Review for the Production of Antidiabetic Peptides by a Bibliometric Approach" publicado na revista: Nutrients 2022,14,4275. Disponível em: https://doi.org/ 10.3390/nu14204275 – Artigo que traz uma revisão bibliométrica, que é um resumo de informações publicadas, sobre peptídeos de diferentes origens, com função antidiabética.

III) Capítulo 3 - Artigo original que contempla resultados e discussão dos experimentos: "Bioactive properties of peptide fractions from Brazilian soy protein hydrolysates: *in silico* evaluation and experimental evidence". Artigo que faz uma análise *in silico* de peptídeos de soja comparando com dados experimentais *in vitro* de atividade antioxidante e de inibição de enzimas digestivas (lipase e glicosidase).

IV) Capítulo 4 - Artigo original que contempla resultados e discussão dos experimentos: "Antiproliferative effects in prostate cancer cells of peptide fractions from Brazilian soy protein hydrolysates". Artigo que faz a avaliação da atividade antiproliferativa de peptídeos de soja frente a linhagem celular de câncer de próstata (PC-3).

INTRODUÇÃO

1. INTRODUÇÃO

A soja (*Glycine max*) é a leguminosa economicamente mais importante do mundo, fornecendo proteína dietética para milhões de pessoas e ingredientes para centenas de produtos. Atualmente, existem inúmeros produtos comerciais à base de soja ou que contêm ingredientes de soja (Yi et al., 2020). A soja é uma fonte abundante e relativamente barata de proteína com alto valor nutricional e excelentes propriedades funcionais (Singh e Hati, 2014). Com a crescente adoção de estilos de vida vegetarianos, uma grande variedade de produtos alimentícios à base de soja tornou-se mais disponível nos supermercados. Além da demanda do mercado, uma razão para tal popularidade pode depender das propriedades nutricionais e versáteis dos grãos de soja, que são adequados para alimentação, dentre os quais estão os peptídeos bioativos (Barati et al., 2020).

Peptídeos bioativos (PB) são compostos orgânicos formados por aminoácidos (AAs) unidos por ligações peptídicas. A sequência de AAs determina a função dos PBs, uma vez que os peptídeos são liberados da proteína-mãe onde estão encriptados. Muitos PBs compartilham algumas características estruturais, incluindo um comprimento de resíduo peptídico entre 2 e 20 AAs e a presença de AAs hidrofóbicos, além de resíduos de arginina, lisina ou prolina. Os efeitos fisiológicos dos PBs incluem propriedades anti-hipertensivas, antioxidantes, anti-inflamatórias, antiaterogênicas, opióides, antimicrobianas, antitrombóticas, imunomoduladoras e anticâncer (Barati et al., 2020; Jahandideh et al. 2022).

Peptídeos podem ser obtidos através de hidrólise enzimática, fermentação ou síntese química, sendo a hidrólise enzimática a mais utilizada (Chelliah et al., 2021) devido ao curto tempo de reação, facilidade de aumento de escala e previsibilidade. Mais de 3.000 peptídeos bioativos diferentes estão relatados no banco de dados BIOPEP-UMW, principalmente de fontes alimentares, e a pesquisa sobre eles continua a crescer (Yang et al., 2021). PBs podem exercer seus efeitos benéficos através de sua captação no lado apical da camada de células epiteliais polarizadas do intestino delgado superior ou pela ativação de diferentes vias de sinalização metabólica e sensorial (Cruz-Casas et al., 2021).

O objetivo principal do presente estudo foi a identificação de peptídeos de concentrado proteico de soja hidrolisado com a enzima papaína e investigação das suas bioatividades antioxidante, anticâncer e antidiabética.

CAPÍTULO I

SOJA E SEUS PEPTÍDEOS BIOATIVOS – UMA BREVE REVISÃO

SOYBEAN AND ITS BIOACTIVE PEPTIDES – A BRIEF REVIEW

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SOJA E SEUS PEPTÍDEOS BIOATIVOS – UMA BREVE REVISÃO

SOYBEAN AND ITS BIOACTIVE PEPTIDES – A BRIEF REVIEW

1.1 – História e produção

Originária da China, a soja (*Glycine max*) foi introduzida na agricultura há mais de 5.000 anos, como alternativa ao abate de animais, mas só foi definitivamente domesticada no século XI a.C. Chegou ao Ocidente apenas no século XVI, com as grandes navegações europeias. O cultivo comercial se deu no início do século XX, nos Estados Unidos e por volta de 1920, o teor de óleo de cerca de 20% e de proteína, cerca de 40% do grão, começou a chamar a atenção das indústrias mundiais, passando a ser um item relevante de comércio exterior. Atualmente a soja é largamente usada na alimentação humana, na forma de óleo, grão, farelo e alimentos processados e na ração animal, para bovinos, suínos e aves (Coelho, 2018, Pipolo e Mandarino, 2016)

A soja representa a principal oleaginosa anual produzida e consumida no mundo e é o principal produto do agronegócio brasileiro. Até abril/22 a estimativa da produção nacional de grãos na safra 2021/2022 era de 122.431 milhões de toneladas em uma área de 40 milhões hectares, variação para mais de aproximadamente 15% em relação à safra do ano anterior, segundo dados do CONAB (2022). Segundo levantamento da Embrapa/Soja, o Brasil continua sendo o maior produtor de grão de soja do mundo, à frente dos Estados Unidos, posição alcançada nos últimos cinco anos. A produção de soja está entre as atividades econômicas que mais apresentam resultados expressivos de crescimento no mercado. Isso se deve à consolidação da oleaginosa como importante fonte de proteína vegetal, contribuindo ativamente para o desenvolvimento econômico-social de várias regiões do país como Mato Grosso, Rio Grande do Sul e Paraná, e principalmente, ao desenvolvimento e estruturação de um sólido mercado internacional (Seixas et al., 2020; CONAB, 2022).

1.2 – Composição

Segundo levantamento bibliográfico recente, a composição do grão de soja é majoritariamente de proteínas, seguida de aproximadamente 20% de lipídios ricos em ácidos graxos insaturados, como ácido linoleico (55%), ácido oleico (21,5%); ácido

linolênico (8%); ácidos graxos saturados (15%) como o ácido palmítico (11%) e o ácido esteárico (4%) e 17% de fibras tanto insolúveis como solúveis (Dalpizol, et al., 2020).

No Brasil, o teor de proteína encontrado em cultivares de soja geralmente é em torno de 40%, porém, segundo Capelin (2021), este valor pode variar dependendo da região e, de acordo com sua forma de cultivo, pode haver ainda grandes diferenças na composição do óleo. Outros fatores como clima, solo e nível hídrico afetam diretamente o teor de proteínas dos grãos de soja. A soja tem um alto valor nutricional e funcional, é a principal fonte vegetal de proteína e óleo, que estão diretamente relacionados ao potencial fisiológico das sementes. Comumente o óleo é o mais utilizado para a alimentação humana sendo extraído por solventes; e a parte sólida, restante do processo de extração do óleo, por ser muito nutritiva é utilizada na produção de rações destinadas à alimentação de animais (aves, suínos e gado de corte) (Custodio, 2003).

A soja é normalmente processada para obtenção de isolados proteicos ou outros produtos finais, como “leite” de soja e tofu, e é uma leguminosa bastante conhecida por suas propriedades benéficas. Por outro lado, os subprodutos do processamento de alimentos vegetais representam um importante problema de descarte para a indústria de óleos, mas também são fontes promissoras de compostos que podem ser utilizados devido às suas propriedades tecnológicas ou nutricionais favoráveis, e hoje são considerados como uma possível fonte de compostos funcionais (Mateos-Aparicio et al., 2010).

O farelo, rico em proteína, é utilizado principalmente na indústria de rações para aves, suínos e bovinos, sendo esse o principal emprego econômico do farelo de soja. O concentrado proteico de soja (CPS), produzido para consumo humano, é obtido de grãos de alta qualidade, limpos e descascados, sendo removida a maior parte do óleo e de substâncias não proteicas e deve conter, no mínimo, 70% de proteína bruta, segundo a Resolução da ANVISA, 1978. Em seu processamento, o solvente é removido sem alterar as características funcionais das proteínas da soja por secagem em temperaturas baixas, gerando os chamados “flocos brancos”. O método mais utilizado para o processamento do CPS é o método de extração, que é caracterizado, basicamente, pelo tratamento dos flocos brancos desengordurados de soja com solução aquosa de etanol a 70-90%; lavagem com água em pH=4,5 ou desnaturação proteica com calor úmido, antes da extração com água. São removidos durante o processo aproximadamente 90% dos oligossacarídeos da soja e uma parte dos fatores anti-nutricionais, como inibidores de tripsina, fitatos e lipoxigenases (Felix, 2011; Thrane et al., 2017; Aires, 2021). A

remoção de carboidratos solúveis (principalmente sacarose, estaquiose e rafinose) aumenta o teor de proteína para > 65%, em base seca. Este é o padrão comercial para proteínas vendidas como concentrados de soja. As propriedades funcionais dos concentrados são modificadas por neutralização (no caso do processo ácido), injeção de vapor e cisalhamento mecânico por homogeneização. Os concentrados de soja, que são modificados, apresentam alta retenção de água e propriedades emulsificantes. Estes concentrados modificados foram descritos como concentrados funcionais. Os concentrados de soja são pulverizados em um pó fluido como uma etapa final do processo (Thrane et al., 2017). Como consequência, a digestibilidade de nutrientes do CPS é maior em comparação ao farelo de soja. O CPS tem alto poder emulsificante e é bom adsorvente de gorduras, possui alta solubilidade e dispersibilidade. A sua utilização na indústria alimentícia é amplamente difundida como fortificante proteico e base nutricional de alimentos liofilizados (Dalpizol, et al., 2020).

1.3 – Proteínas da soja

As principais proteínas de soja são conhecidas como β-conglicinina e glicinina e respondem por de 65% a 80% das proteínas totais, são globulinas solúveis em soluções salinas.

A glicinina (também chamada de 11S Globulina) é a maior fração da proteína total da semente de soja (25-35%), tem um alto peso molecular (320-375 kDa) e consiste em seis subunidades ácidas e seis básicas dispostas em uma estrutura hexamérica. As subunidades são mantidas juntas por ligações dissulfeto. A β-Conglicinina (também chamada de 7S Globulina) é uma proteína trimérica com cerca de metade do peso molecular da glicinina (175 kDa). As três subunidades (β, α e α') apresentam massa molecular relativa de, respectivamente 50, 67 e 71 kDa, mas podem variar de acordo com a variedade do grão. Todos os três tipos de subunidades são glicoproteínas com 4-5% de carboidratos. As propriedades da β-conglicinina são alta viscosidade e baixa estabilidade térmica. Devido à composição e estrutura diferentes, essas globulinas de soja têm efeitos compensatórios nas propriedades nutricionais e funcionais dos ingredientes de proteína de soja (Liu, 1999; Thrane et al., 2017; Preece et al., 2017).

Outras proteínas metabólicas dentro da soja incluem oleosinas (8-20% da proteína total) para estabilização dos corpos de óleo, inibidores de tripsina (0-1,7% da

proteína total) e enzimas como a lipoxigenase (LOX). Tanto os inibidores de tripsina quanto a LOX são inativados depois de tratamento térmico (90% de inativação após 5–10 min a 121 °C a pH 6,5), impedindo que formem complexos estáveis com as tripsinas e quimotripsinas no trato gastrointestinal e ativem a oxidação de ácidos graxos poli-insaturados e a formação de hidroperóxidos de ácidos graxos, que geram aromas desagradáveis, respectivamente (Preece et al., 2017). Estudos com melhoramento genético identificaram a possibilidade de variantes de soja livres de LOX e podem ser usados para redução do sabor de soja sem restrição por órgãos regulatórios que governam organismos transgênicos (Wang et al., 2019).

O padrão atual para avaliar a qualidade da proteína é o escore de aminoácidos indispensáveis digestíveis (DIAAS), que foi cunhado após abordar algumas limitações do antigo escore de aminoácidos corrigidos pela digestibilidade da proteína (PDCAAS). Uma grande diferença entre esses dois escores de qualidade de proteína é que o DIAAS relaciona a quantidade de proteína ingerida com os níveis presentes no final do intestino delgado ("digestibilidade ileal"), enquanto o PDCAAS usa os níveis de proteína remanescente nas fezes ("digestibilidade fecal"). van den Berg e colaboradores (2022) revisaram as quantidades de PDCAAS e DIAAS da soja e seus subprodutos, como farelo e isolado, e observaram que a maioria dos produtos de soja tem altas pontuações de qualidade de proteína e que as condições de processamento e pós-processamento podem aumentar ou diminuir a qualidade da proteína.

1.3.1 – Alergenicidade

Segundo a OMS/IUIS (International Union of Immunological Societies), existem registradas oito proteínas na soja que podem provocar uma resposta alérgica (www.allergen.org). Duas delas, β-conglicinina e glicinina, representam aproximadamente 70% da proteína da soja, como já citado acima e, consequentemente, serão as proteínas predominantes em produtos comerciais de proteína de soja. A alergia à soja pode causar sintomas alérgicos como asma, choque e morte. Assim, identificar alérgenos e reduzir a alergenicidade são cruciais para resolver o problema das alergias à soja. Estudos tem demonstrado que processamentos adicionais, como térmico, hidrólise enzimática, fermentação, ultrassom e tratamento com plasma frio, podem reduzir a alergenicidade da soja resultando na destruição de epítopos e na redução de alérgenos principalmente pela mudança estrutural das proteínas. Como resultado, o peso

molecular é reduzido e a imunogenicidade das proteínas alimentares é também reduzida. A combinação de vários métodos pode ser uma alternativa potencial para reduzir a alergenicidade da soja e uma aplicação de rotulagem adequada é vital para garantir que os consumidores suscetíveis evitem a ingestão (Wang et al., 2022; Oyedeji et al., 2018; Thrane et al., 2017; Mulalapele e Xi, 2021). Segundo Peighambardoust e colaboradores (2021), as proteínas presentes nos alimentos são suscetíveis a alterações, levando à criação de peptídeos alergênicos; formação de compostos derivados como nitrosaminas, acrilamida e aminas biogênicas como resultado de interações de peptídeos com a matriz alimentar durante o processamento e armazenamento, e mais estudos são necessários em relação à alergenicidade de peptídeos gerados, não apenas os provenientes de soja.

1.3.2 – Formas de consumo da soja

O consumo de soja e produtos de soja foi inversamente associado com mortes por câncer e doenças cardiovasculares em uma meta-análise realizada com 23 estudos, incluindo 330.826 participantes (Nachvak et al., 2019). Nos últimos anos tem sido crescente o número de pessoas que aderem a dieta vegana/vegetariana tendo a proteína de soja como uma opção de substituição a produtos de origem animal, assim como alérgicos e intolerantes à lactose que não consomem laticínios e derivados, tendo produtos a base de soja como substitutos para a sua dieta, pois são isentos de colesterol, glúten e lactose (Paul et al., 2019; Munekata,et al., 2020; Ashaolu, 2020)

A soja e seus derivados, como a farinha, a proteína texturizada ou “carne” de soja e o extrato ou “leite” de soja, quando utilizados em alimentos associados aos cereais, como trigo, milho e centeio, conferem um bom balanço de aminoácidos essenciais, como isoleucina, leucina, lisina e triptofano (Carrão-Panizzi e Mandarino, 1998). Segundo Gregory Ribeiro, no último Dairy Vision (2021), o Brasil apresentou crescimento de 13% no consumo de produtos *plant-based* impulsionado pela ausência de lactose (Milkpoint, 2021). A bebida vegetal à base de soja foi a que apresentou maior conteúdo de proteínas (2,7 g/100mL), quando comparada a amêndoa, castanha de caju, arroz e coco (1,0; 1,5; 0,6 e 0,3 g/100mL, respectivamente), estando mais próxima da quantidade de proteína obtida no leite de vaca (3,1 g/100mL) (Mello et al., 2021). Apesar disso, deve ser considerada com atenção, em virtude da ausência de vitamina D e do baixo conteúdo de cálcio, comparado ao leite de vaca, fonte desses nutrientes (Munekata et al., 2020).

1.3.3 – Potenciais efeitos benéficos da soja e seus peptídeos

A soja é considerada um alimento funcional, isto é, capaz de produzir efeitos benéficos à saúde, além de suas funções nutricionais básicas e de seu valor nutritivo, inerente à sua composição química. Esta classificação se dá principalmente pela presença de proteínas e isoflavonas que possuem ação estrogênica e ação anticâncer (BRASIL, 2022). Por sua composição, também parece ter efeito positivo sobre a osteoporose e sintomas da menopausa (Messina, 2014).

Outra forma de se explorar as funcionalidades das proteínas de soja é a geração de peptídeos biologicamente ativos a partir de proteínas precursoras. Esses peptídeos podem ser obtidos de várias maneiras, incluindo hidrólise enzimática: pelas enzimas digestivas animais ou pelas enzimas derivadas de microrganismos e de plantas, processamento de alimentos: maturação, fermentação, cozimento e armazenamento.

Diferentes estudos têm demonstrado que o consumo de peptídeos de soja tem vários efeitos positivos para a saúde, incluindo atividades antioxidantes e anti-hipertensivas, inibição de enzimas digestivas, redução do colesterol e da gordura corporal e redução da incidência de câncer de estômago, colorretal e mama (Coscueta et al., 2019; Wang et al., 2019, Martinez-Villaluenga et al., 2010, Pak et al., 2005).

1.3.4 – Potenciais aplicações

O uso de ingredientes de proteína de soja para substituir proteínas de aves, carnes, peixes, ovos e laticínios pode melhorar a qualidade dos produtos alimentícios, reduzir os custos de várias formulações alimentícias e melhorar a sustentabilidade ambiental associada ao fornecimento de proteína de qualidade (Thrane et al., 2017). Dentre as principais propriedades dos hidrolisados de soja podemos destacar solubilidade, gelificação, emulsificação, formação de espuma, capacidade de retenção de gordura e água, potencializando as propriedades reológicas e ponto de fusão de alimentos à base de emulsão, como sorvetes (Ashaolu, 2020).

A expansão do uso de proteínas de soja em produtos cárneos é limitada devido aos sabores indesejáveis amargos e a sensação bucal adstringente. Estudos sensoriais têm demonstrado que o concentrado proteico de soja pode ser utilizado no teor máximo de 3% sem alterar significativamente o sabor característico do produto pelo sabor indesejado da soja (Moraes et al., 2009). Alguns métodos eficientes para melhorar o

sabor de peptídeos ativos foram resumidos em um artigo de revisão recente (Fu et al., 2019). Diferentes soluções têm sido desenvolvidas para reduzir o amargor, como o uso de enzimas para reduzir o teor de peptídeos amargos. A "remoção" desses peptídeos se dá por meio de técnicas específicas (separação em gel, extração em álcool, cromatografia em sílica gel e precipitação isoelétrica) individualmente ou em combinação, e a transformação, modulação ou mascaramento do sabor, através do uso de agentes modificadores de sabor, como açúcares, sais e nucleotídeos ou através da fermentação do produto, uma vez que, durante este processo o sabor se altera (Cruz-Casas et al.,2021).

De acordo com Pereira e colaboradores (2011), a adição de isolado de proteína de soja em sorvete reduziu o efeito do choque térmico na recristalização do gelo, na taxa de derretimento e aumentou a quantidade de cristais pequenos de gelo, além disso, pôde ser incorporado na formulação de sorvetes até o nível de substituição de 20% sem afetar sua aceitação sensorial. Outro estudo com sorvete, onde os hidrolisados de proteína de soja preparados com as enzimas papaína e pepsina foram utilizados, tiveram alteração positiva nas propriedades interfaciais e viscoelásticas e demonstraram excelente estabilidade da emulsão e taxa de fusão, uma vez que a subunidade β, da β-conglicinina não pode ser deslocada pelos monoglicerídeos, levando à inibição da coalescência de gordura no sorvete (Chen et al., 2019).

1.4- Peptídeos de soja

Peptídeos bioativos são fragmentos de proteínas específicas, geralmente compostos de 2 a 20 resíduos de aminoácidos, possuem peso molecular inferior a 6.000 Da, e se destacam por promover efeitos positivos à saúde do consumidor. Peptídeos bioativos da soja estão associados a importantes atividades biológicas como anti-hipertensiva, antioxidante, anti-inflamatória, imunomoduladora, hipolipidemiante, antidiabética, anticancerígena etc. (Cruz-Casas et al., 2021). Eles podem ser obtidos através de hidrolise enzimática, fermentação microbiana ou por síntese química (Yang et al., 2021). A Tabela 1 mostra uma revisão sobre os principais trabalhos recentes com peptídeos obtidos tendo a proteína de soja como matriz.

Tipo de proteína	Sequência responsável	Enzima	Atividade	Referência
Leite de soja		Papaína	Antioxidante e antimicrobiana	Nath, 2020

Hidrolisado proteico de soja	VVFVDRL (847 Da, SPH-IA), VIYVVDLR (976 Da, SPH-IB), IYVVDLR (877 Da, SPH-IC), IYVFVR (795 Da, SPH-ID)	Alcalase	Antioxidante e cito protetiva (CaCo2)	Zhang 2019
Leite de porca enriquecido com SPH			Melhor a concentração de aminoácidos funcionais e as propriedades antioxidantes em leitões recém-nascidos.	Zhuo, 2020
Isolado proteico de soja		Bromelina, papaína, pepsina, tripsina, neutral protease e alkaline protease	Forte atividade antioxidante contra estresse oxidativo induzido por H_2O_2 em células HepG2	Yi 2020
Torta de soja preta	Leu/Ile-Val-Pro-Lys (L/I-VPK)	Alcalase	Antioxidante e anticancerígena (fígado (HepG2), pulmão (MCF-7) e cervical (Hela))	Chen 2018
Hidrolisado proteico de soja	IRHFNEGDVLVIPPGVPY, IRHFNEGDVLVIPPGVPYW, IYNFREGDLIAVPTG, VSIIDTNSLENQLDQMPRR, YRAELSEQDIFVIPAG.	Corolase PP	Antioxidante e inibição de ECA	Coscueta 2019
Produto de soja fermentado			Antioxidante e peroxidação lipídica	Tonolo 2019
Peptídeos sintéticos	Lunasina e derivados		Anti-hipertensiva, antioxidante e anti oxidativa	Indiano-Romacho, 2019
Hidrolisados de soja verde		Alcalase	Inibição de ECA	Hanafi, 2018
Soja preta		*Bacillus* spp.	Atividade antioxidante	Sanjukta 2021
Hidrolisados de soja		Alcalase	Atividade imunomoduladora	Wen 2021
Isolado proteico de soja	WPK e AYLH	Alcalase e flavorzyme (3:1)	Anti-idade e antioxidante	Amakye 2021
Farinha de soja		α- quimotripsina	Antioxidante	Cavalieri 2021
Soja fermentada		*Bacillus*, *Lactobacillus* e *Hansenula* strains	Antioxidante e anti-fadiga (*in vitro* e *in vivo*)	Cui 2020
Leite de soja			Antioxidante, inibidor de ECA e antibacteriana	Singh 2020
Okara		Alcalase	Antioxidante e resposta imune	Fang 2021
Isolado proteico de soja		Alcalase	Antioxidante	Zhang 2021
Lunasina			Antioxidante, anticancerígena e anti-inflamatório	Hao 2020
Soja germinada	NDDRDS, VVNPDNNEN, LSSTEAQQS, NAENNQRN, IKSQSES, EEPQQPQQ, QSSRPQD, LAGNQEQE, NLKSQQA, QEPQESQQ, SQRPQDRHQ, QQQQQGGSQSQ, QQQQQGGSQSQKG, PETMQQQQQQ, e SDESTESETEQA		Anticâncer	Gonzalez-Montoya 2018

Soja germinada	>10 kDa, 5– 10 kDa e <5 kDa		Antioxidante e anticâncer	Gonzalez-Montoya 2016
Tempeh	Ile-Arg	*In silico*	Anticâncer	Tamam 2020

Estudos de revisão recentes na literatura são encontrados demonstrando a estrutura, obtenção e biofuncionalidades de peptídeos não só de soja, mas também de outras matrizes alimentares (Yang et al., 2021; Chelliah et al., 2021; Cruz-Casas et al., 2021). Visto isto, estudos com a enzima papaína são ainda escassos na literatura apesar dessa hidrólise trazer resultados satisfatórios, como é o caso de Nath e colaboradores (2020), onde a hidrólise de leite de soja por 10 minutos apresentou bom grau de hidrólise e atividade antioxidante dose-dependente da quantidade de enzima, devido ao fato de que com o aumento da concentração de papaína, maiores quantidades de ligações peptídicas no leite de soja foram hidrolisadas e aminoácidos hidrofóbicos foram expostos.

A atividade antioxidante geralmente é a mais estudada quando se fala sobre peptídeos de soja. Em geral, os peptídeos de baixo peso molecular apresentam maior atividade antioxidante do que suas proteínas originais, porque são mais acessíveis aos radicais livres (Yang et al., 2021). Em estudo de Coscueta e colaboradores (2019), os hidrolisados proteicos de soja tiveram atividade antioxidante mesmo após a digestão simulada *in vitro*, e após análise de massa molecular desses peptídeos, foi identificada compatibilidade com a absorção pelo lúmen intestinal, permitindo assim que os peptídeos exerçam uma função nutricional ou bioativa. Hidrolisados de soja também apresentaram boa atividade antioxidante em estudos celulares na prevenção da formação de radicais livres após sofrerem ação gastrointestinal (Cavaliere et al., 2021). Sabe-se pela literatura que aminoácidos hidrofóbicos e antioxidantes, como Val (V), Leu (L), Ile (I), Phe (F), Tyr (Y) e Asp (D), contribuem significativamente para as atividades de eliminação de radicais livres (Yang et al., 2021; Sompinit et al., 2020) aumentando suas interações com lipídios ou atuando como potentes doadores de prótons/hidrogênio, o que foi evidenciado também em estudo recentes (Zhang et al., 2019; Zhang et al., 2021). Zhuo e colaboradores (2020) indicaram que a suplementação da alimentação de porcas gestantes com peptídeos de soja melhorou o desempenho de crescimento de leitões lactentes, melhorando a concentração de aminoácidos funcionais e propriedades antioxidantes.

Nos últimos anos, tem sido relatado o potencial anti-hipertensivo das frações proteicas da soja, juntamente com o isolamento e caracterização estrutural dos peptídeos mais ativos, principalmente pela inibição de uma enzima chamada ECA (enzima conversora de angiotensina -I), que tem papel fundamental no controle da hipertensão. Singh e colaboradores (2020) trabalhando com leite de soja fermentada, tiveram atividade de inibição de ECA significativamente maior quando comparado ao leite não fermentado, indicando que alguns peptídeos inibidores da ECA foram liberados durante a fermentação. Outra forma de obtenção de peptídeos anti-hipertensivos é a hidrólise enzimática. No trabalho de Hanafi e colaboradores (2018) os hidrolisados de soja verde com a enzima Alcalase apresentaram alta atividade inibitória da ECA, variando de 90,4% a 100% de inibição, sugerindo que esses hidrolisados poderiam potencialmente se tornar ingredientes no desenvolvimento de alimentos funcionais.

Os primeiros estudos com proteínas de soja identificaram o peptídeo lunasina (43 aminoácidos, 5.5 KDa) como o principal responsável pela atividade anticancerígena em 1987. A partir de então começaram as buscas para avaliar as propriedades anticancerígenas dos peptídeos de soja. Chen e colaboradores (2018) identificaram e purificaram um tetrapeptídeo a partir de farelo de soja preta (L/IVPK) e revelaram que tanto os efeitos hidrofóbicos quanto as ligações de hidrogênio entre peptídeos purificados e proteínas-alvo podem ser responsáveis pela potente atividade anticancerígena verificada em linhagens de câncer de fígado, de pulmão e cervical. Em revisão recente sobre peptídeos de grãos com ação antitumoral em células de câncer de colón, identificou-se atividade de peptídeos de soja nas linhagens HCT-116, Caco-2 e HT-29, além da ação da lunasina com parada do ciclo celular em G1 e apoptose (ativação de caspase-3 e clivagem de Poli (ADP-ribose) polimerase) (Avilés-Guaxiola et al., 2020).

Alguns peptídeos alimentares demonstraram desencadear dois ou mais papéis fisiológicos, portanto, a bioatividade em uma região frequentemente resulta em um efeito benéfico em outra parte dos sistemas do organismo. Assim, as bioatividades e seus efeitos em várias partes de um sistema de corpo vivo estão interligados. Além disso, como a maioria das doenças apresenta mais de um sintoma, os peptídeos multifuncionais podem ser usados para atingir algumas das situações patológicas simultaneamente (Indiano-Romacho et al., 2019). Estudos *in vitro* e *in vivo* devem ser cada vez mais desenvolvidos para o entendimento das ações desses peptídeos no organismo dos pacientes.

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CAPÍTULO II

Critical Review for the Production of Antidiabetic Peptides by a Bibliometric Approach

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Abstract

The current bibliometric review evaluated recent papers that researched dietary protein sources to generate antidiabetic bioactive peptides/hydrolysates for the management of diabetes. Scopus and PubMed databases were searched to extract bibliometric data and, after a systematic four-step process was performed to select the articles, 75 papers were included in this review. The countries of origin of the authors who published the most were China (67%); Ireland (59%); and Spain (37%). The journals that published most articles on the subject were Food Chemistry (n = 12); Food & Function (n = 8); and Food Research International (n = 6). The most used keywords were 'bioactive peptides' (occurrence 28) and 'antidiabetic' (occurrence 10). The most used enzymes were Alcalase® (17%), Trypsin (17%), Pepsin, and Flavourzyme® (15%each).It was found that different sources of protein have been used to generate dipeptidyl peptidase IV (DPP-IV), α-amylase, and α- glucosidase inhibitory peptides. In addition to antidiabetic properties, some articles (n = 30) carried out studies on multifunctional bioactive peptides, and the most cited were reported to have antioxidant and antihypertensive activities (n = 19 and 17, respectively). The present review intended to offer bibliometric data on the most recent research on the production of antidiabetic peptides from dietary proteins to those interested in their obtention to act as hypoglycemic functional ingredients. The studies available in this period, compiled, are not yet enough to point out the best strategies for the production of antidiabetic peptides from food proteins and a more systematic effort in this direction is necessary to allow a future scale-up for the production of these possible functional ingredients.

Keywords: hypoglycemic bioactive peptides; protein hydrolysis; DPP-IV; α-amylase; α-glucosidase; inhibitory peptides

1. Introduction

Diabetes is an old disease of which there are at least three known types: type 1 usually occurs during childhood and adolescence, as a result of the failure of the β-cells in the pancreatic islets to secrete insulin, caused by an autoimmune condition; type 2 is the most common and is usually due to eating habits and lifestyle; and there is also gestational diabetes. It is estimated that type 2 diabetes (T2D) accounts for more than 90% of the total cases, thus, the research for strategies to prevent, cure, or slow down the progress of this disease is a subject of interest to the scientific community. In 2017, approximately 462 million individuals were affected by T2D, which corresponds to 6.28% of the world population, and this number is projected to increase to 700 million by 2045. It is estimated that this disease and its complications caused 4.2 million deaths globally in 2019, making it the ninth leading cause of mortality worldwide, and its prevalence has been growing faster in low- and middle-income countries than in their high-income counterparts. The fraction of the adult population with diabetes in 2019 in high-income countries was 10.4%, followed by middle-income countries with 9.5%, whereas low-income countries showed the lowest prevalence (4.0%). Prevalence is projected to increase in the three income groups to 11.9%, 11.8%, and 4.7%, respectively, by 2045.

Diabetes mellitus is a metabolic disorder that is a worldwide public health concern and poses significant economic and social challenges [1]. Research has been confirming that food protein hydrolysates with *in vitro* DPP-IV, α-amylase, and α-glucosidase inhibitory properties are potential agents against T2D [2]. Moreover, the American Diabetes Association and the European Association for the Study of Diabetes have approved the use of these hydrolysates as antihyperglycemic drugs [3]. Thus, the development of potent antidiabetic hydrolysates containing bioactive peptides with specific inhibitory activity is of great scientific interest [2].

Treatment against T2D consists of lifestyle modification and the use of drugs that increase glucose uptake in tissues, decrease gluconeogenesis, or stimulate insulin secretion. A relatively novel treatment involves dipeptidyl peptidase IV (DPP-IV), α-glucosidase, and/or α-amylase inhibitors that display a hypoglycemic effect by reducing intestinal glucose absorption and enhancing the synthesis of insulin, as well as acting as

receptor agonists of glucagon-like peptide-1 (GLP-1) [1; 4]. At present, there is no cure for diabetes mellitus and the use of synthetic drugs that may have side effects is a widely applied treatment. The hazards associated with these therapies include hypoglycemia, weight gain, tiredness, diarrhea, and anemia, among others [5]. Therefore, the development of natural food-derived peptide remedies may not only avoid the side effects, but also allow the early intervention and nutritional treatment of patients. Thus, they may be the ideal choice for preventing diabetes and improving its treatment [6]. To be effective, peptides need to be released from the protein matrix, be bioaccessible, and reach the active site in sufficient quantities to exhibit biological activity. Recently, several studies have been focusing on the generation of bioactive peptides from food proteins and their utilization as functional ingredients.

Literature reviews play an essential role in academic and technological research, gathering available knowledge, and pointing out the state of the art in a given field. The compilation of academic knowledge grows challenging, as scientific knowledge keeps increasing exponentially and thousands of new articles are published on a daily basis. This task becomes even more troublesome with the advent of predatory journals publishing open access articles without proper peer review. Bibliometric reviews can help to overcome several difficulties encountered in collecting reliable bibliographic data: separating what has already been tested from what still needs to be tested or better evaluated, relating methods used and the results obtained, and indicating what is known and what is not known in that specific field, in a quantitative way [7]. The goal of the present study was, therefore, to carry out a quantitative survey of the knowledge available in reliable databases about the factors involved in the production of peptides with antidiabetic activity in order to provide an overview of what has already been successfully tested, what needs to be further evaluated, and, by exclusion, what has not yet been studied in this topic.

2. Methodology

2.1. Search Strategy of the Bibliometric Analysis

The bibliometric review was based on the methodological research proposed by Randhawa, Wilden, and Hohberger [8]. The search approach was conducted using ‘Scopus’, a major citation database of peer-reviewed literature, and ‘PubMed’, an index of biomedical literature, including all data available in the ‘Medline’ database. The

search included published papers (original and review papers) that had focused on protein hydrolysates containing bioactive peptides with antidiabetic properties that could be applied to functional foods and nutraceuticals.

The first step consisted in identifying the descriptors to define three cores: (i) the active principle, by including 'bioactive peptide' or 'inhibitory peptide', (ii) the type of inhibition, and (iii) the expected bioactivity. For the 'Scopus' database search, the fol-lowing descriptors were used: ("bioactive peptide" OR "inhibitory peptide") AND ("alpha glucosidase" OR "alpha amylase" OR "dipeptidyl peptidase" OR "DDP-IV") AND ("antidiabetic" OR "anti-diabetic"). The same descriptors were applied for the 'PubMed' database search, however, without the use of quotes framing each keyword.

2.2. Review Process and Selection Criteria

In the next step, two authors, working independently, identified the relevance of the papers by reviewing the titles, abstracts, and keywords that appeared in each search. The publication period taken into consideration for the present review was between 2016 and May 2021, and the whole search and selection process was performed between 21 May to 28 May 2021. All the selected papers were reviewed to avoid any loss of crucial references. An article was included for the analysis only if after screening: (i) it was published between 2016 and 2021 (ii) it was a peer-reviewed research or review article; (iii) it was in the final publication stage; (iv) it was published in English; (v) and that there was a full text available. While the records excluded were (i) non-research articles (e.g., book chapter, short survey, documents); (ii) there was any missing data in the article (e.g., authors' name); (iii) if it was not published, it means that articles in press were not con-sidered; (iv) articles published in a different language of English (e.g., Chinese); (v) ar-ticles that the full text was not available (e.g., only the abstract available); (vi) non-peer-reviewed articles; (vii) and off-topic studies (e.g., those who did not make enzymatic hydrolysis to generate bioactive peptides).

2.3. Data Extraction

The data extracted for each individual study included the following: database that the article was identified; keywords; country of publication; year; journal of the publication; type of the article (e.g., original or review); inhibition type; multifunction

peptides assays; enzyme used; source of protein used; type of analysis (e.g., *in vitro*, *in vivo*, or *in silico*); peptide sequence; outcomes; and challenges and perspectives.

3. Results and Discussion

This bibliometric review intended to provide a survey of recent articles that presented research on antidiabetic bioactive peptides from different dietary protein sources to be used as food ingredients and improve glycemic regulation of diabetic patients.

In Figure 1, a flow chart shows the records identified in each database and the total number of articles (n = 386); the records that were screened and the eligible ones; and the total number of studies included in this review (n = 75). After a refined search, 28 papers were found to be present in both databases. Of the total of 75 papers in the final selection, 22% (n = 17) were exclusive to the 'Scopus' database and 40% (n = 30) were exclusive to the 'PubMed' database.

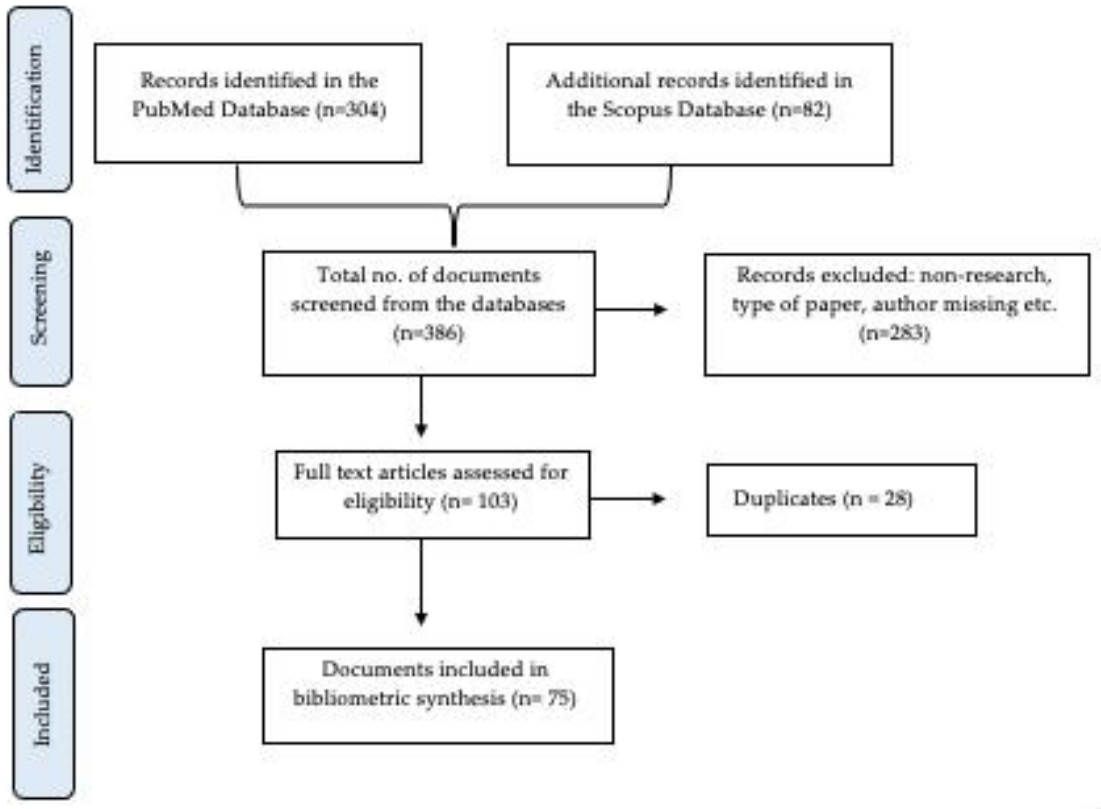

Figure 1. Flowchart indicating included and excluded articles.

3.1. Number of Publications, Authors' Countries/Territories, Publication Period, and Leading Journals

Among the 75 included articles, original articles accounted for 85% (n = 64) of the selected articles during the whole period (2016 to May 2021), and they focused on different methods to obtain peptides from several matrices. Over the target period of publications, there was an increase in the number of identified peptide sequences, with a greater number of peptides reported for the first time in 2020 and 2021. This shows the growing interest in this subject and reinforces the usefulness of compilations such as the present study.

Among the 27 countries that studied antidiabetic peptides, there was a total of 98 researchers who authored the publications. The countries with the highest number of publishing authors were China (n = 18; 67%); followed by Ireland (n = 16; 59%); then Spain (n = 10; 37%). In terms of distribution of published articles per continent, Asia (n = 38; 39%) published the highest number of articles, followed by Europe (n = 34; 35%), the Americas (n = 14; 14%), Africa (n = 8; 8%), and Oceania (n = 4; 4%). Globally, China (116.4 million), India (77.0 million), the USA (31.0 million), Pakistan (19.4 million), Brazil (16.8 million), Mexico (12.8 million), Indonesia (10.7 million), Germany (9.5 million), Egypt (8.9 million), and Bangladesh (8.4 million) were the top 10 countries with the highest number of diabetics in 2019 [9]. These data may explain the interest shown by Chinese as well as other Asian researchers in this topic, but also points to the fact that academic research on antidiabetic peptides is not specifically located in countries with the highest incidence of this disease.

Three journals have published most of the papers about antidiabetic peptides. For these particular journals, there was a correlation between the year of publication and the authors' countries of origin through the Sankey diagram (Figure 2). The journals that most published on subjects related to DPP-IV, α-amylase, and α-glucosidase inhibitory peptides were Food Chemistry (n = 12); followed by Food & Function (n = 8); and then Food Research International (n = 6). When the top publishing journals and the author's countries were correlated, it was found that the country that appeared most frequently, Ireland (n = 12), corresponded for about half of the articles published in these leading journals (total number of published articles in the three leading journals = 26), compared to the United Arab Emirates and Spain (n = 4, each), China (n = 3), Malaysia and the USA (n = 2, each), while the other authors' countries in the diagram

had 1 published article each. Therefore, it appears that although China is the country of origin with the highest overall number of authors/publications, it did not contribute the most to the three main journals when publishing on the subject.

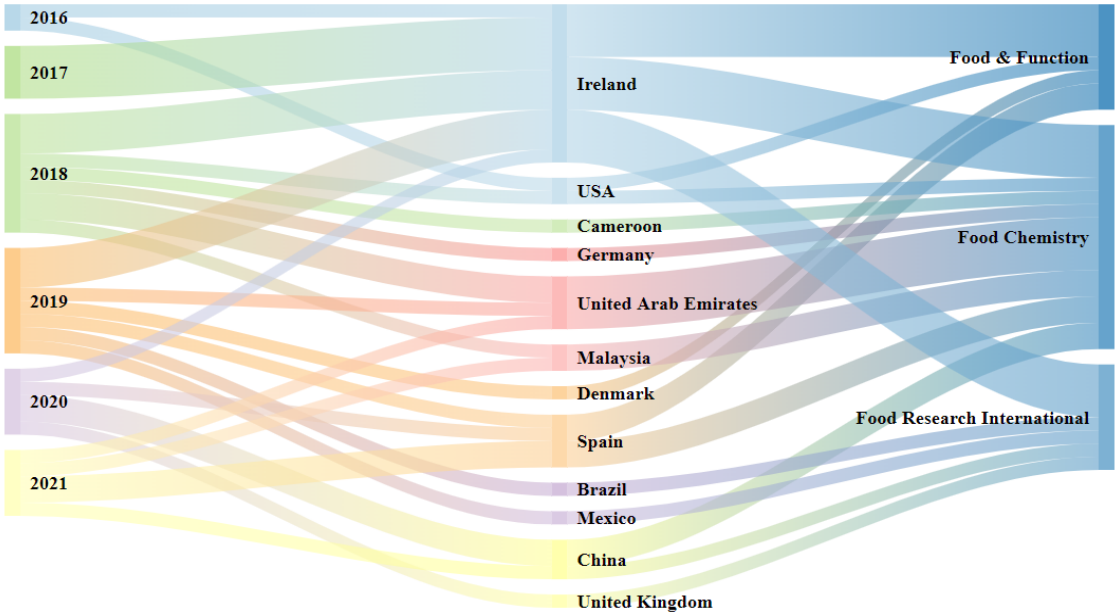

Figure 2. Sankey diagram for the selected articles relating year of publication, authors' country of origin, and the three leading scientific journals that published the antidiabetic bioactive peptide studies.

3.2. Author's Keywords

The author's keywords that appeared among the selected published articles more than twice, were recorded and are shown in Figure 3. Two hundred and eighteen different keywords were found among the included articles, of which 46 appeared at least in two different papers. The most used keywords were: 'bioactive peptides' (occurrence 28, total link strength 54) and 'antidiabetic' (occurrence 10, total link strength 38). When the keywords were grouped according to their meaning, the top five groups were: 'bioactive peptide, bioactive peptides, and bioactivity' (occurrence 38), which also showed up as the main keyword-group followed by 'DPP-IV' and its variations, 'dipeptidyl peptidase IV, dipeptidyl peptidase IV inhibition, dipeptidyl peptidase-IV and DPP-IV inhibition' (occurrence 25); 'antidiabetic, antidiabetic activity and antidiabetic potential' (occurrence 16); 'α-glucosidase, α-glucosidase inhibition, α-glucosidase inhibitory activity' (occurrence 10); and 'protein hydrolysate, and protein hydrolysates' (occurrence 9). As expected, the most cited keywords match the

descriptors used in the bibliometric search. The evaluation of the keywords highlighted that, within the most tested enzymes related to diabetes, DPP-IV was the most represented in the author's keywords, indicating that it has been the most tested, followed by alpha-glucosidase, the second most tested enzyme.

Other groups of keywords were 'antioxidant, and antioxidant activity' (occurrence 11); and 'ACE, ACE inhibition, and angiotensin-converting enzyme' (occurrence 7). These latter keywords appeared very frequently, owing to the multifunctionality of many bioactive peptides, which, in addition to acting as antidiabetic agents, may present other bioactivities, e.g., antioxidant and hypotensive capacities. Other keywords also frequently appeared among the selected articles and their occurrence was mostly related to peptide bioactivities; the methods used to identify these peptides; the diseases that might be suppressed/treated by their use; and the proteins used as a substrate to obtain the hydrolysates.

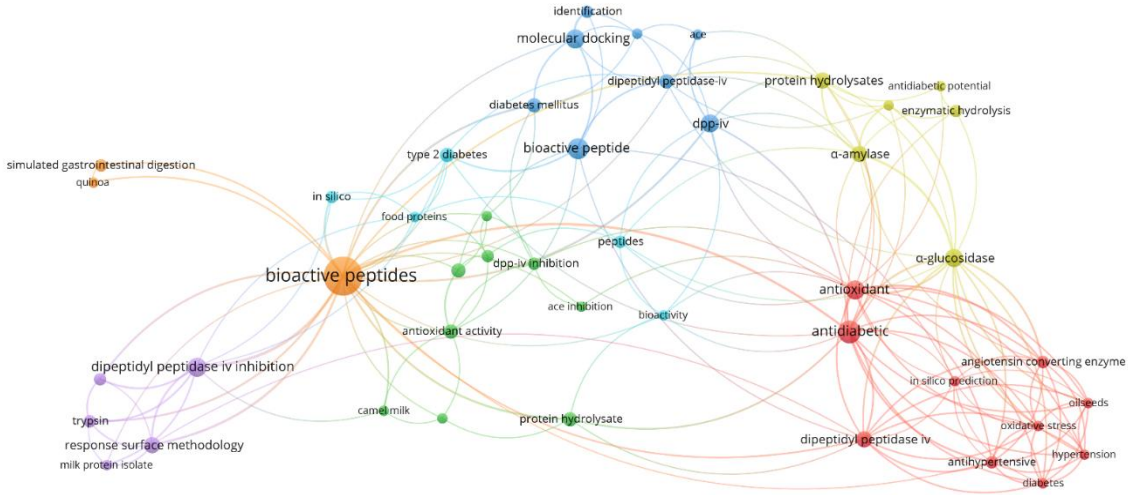

Figure 3. Network visualization map of the articles' keywords that appeared at least twice. Node size is proportional to the number of occurrences (VOSviewer).

3.3. The Most Used Proteases

Bioactive peptides are inactive in the sequence of the native protein molecule; however, they may be released through protein hydrolysis [10]. This reaction is based on the cleavage of the peptide bonds, which generates peptides of different sizes. Depending on the aminoacidic sequence of these peptides, the hydrolysate might present some biological activity [11]. To produce hydrolysates, protein hydrolysis can

be carried out by methods such as chemical treatment; microbial fermentation; or enzymatic hydrolysis, by using proteases. Among these techniques, hydrolysis by proteases is the most frequently used to produce bioactive peptides [12]. Some of the advantages of enzymatic hydrolysis, when compared to chemical hydrolysis or fermentation processes, are: the generation of final products free from toxic compounds and organic solvents; an improved reaction rate; also, this technique is performed under mild temperature and pH conditions; enzymes present high specificity and good stereoselectivity; the reaction normally does not produce secondary products; the use of protease provides an environmentally friendly procedure; enzymatic hydrolysis usually produces higher peptide yields, which also have good quality; enzymes are easy to inactivate; and in general, the process is fairly simple [12,13]. As disadvantages, several parameters, such as enzyme-substrate ratio, hydrolysis time, pH, and reaction temperature, must be monitored and managed in an optimal range during enzymatic hydrolysis, because these factors may affect diverse properties in the resulting peptides. Usually, a pre-treatment of the substrate is necessary to enhance hydrolysis; and, last but not least, this method presents a higher cost, in comparison to other processes such as fermentation, owing to the high price of the enzymes [14].

Twenty-one different enzymes were identified among the selected published articles. The most used enzymes were Alcalase®, also referred to as subtilisin (EC 3.4.21.62) (n = 23; 17%), followed by trypsin (EC 3.4.21.4) (n = 22; 17%), pepsin (EC 3.4.23.1) (n = 20; 15%, each), and Flavourzyme® (a commercial mixture of fugal endo- and exo-peptidases (EC 3.4.11.1)) (n = 15; 11%).

Alcalase® is the trade name of the subtilisin produced by the Novozymes company. It is a microbial peptidase, produced from Bacillus licheniformis [15]. This protease is a serine S8 endo-proteinase that presents broad specificity and prefers to cleave large uncharged residues in the P1 position [13]. Owing to its broad specificity, this enzyme usually generates peptides with low molecular weight [16], which may explain its frequent use, as many peptides that present a variety of bioactivities, including antidiabetic activity, also show low molecular mass [16,17].

Trypsin is an animal protease, and all the studies included in the present review used trypsin extracted from porcine pancreas. This enzyme is an endopeptidase that shows specificity to cleave bonds involving arginine and lysine residues [18]. Its narrow

specificity usually leads to the release of peptides with bioactivities owing to their terminal residues of R or K, which are considered molecular features for peptides that can inhibit α-glucosidases [19]. Trypsin has been used to obtain protein hydrolysates with different industrial uses for some time and presents the advantage of producing peptides possibly resistant to digestion by pancreatic enzymes.

Pepsin used in all papers included here was obtained from porcine gastric mucosa [20]. This protease presents broad specificity and prefers to cleave peptide bonds in-volving aromatic and hydrophobic amino acids [18]. According to Gomez et al. [21], this ability to break aromatic amino acid linkages might be related to increased DPP-IV inhibitory activity, as DPP-IV inhibitory peptides generally show a branched-chain amino acid or an aromatic residue containing a polar group in the side chain at their N-terminal position and/or a proline residue located at P1. Like trypsin, pepsin has several traditional industrial applications and presents the possibility of generating peptides resistant to gastric digestion.

Flavourzyme® is a trademark name given to a mixture of proteolytic enzymes by the Novozymes company. Flavourzyme® is produced by *Aspergillus niger* and exhibits endo- and exopeptidase activities [22]. Owing to its exopeptidase activity, Flavourzyme® is able to release very small peptides and free amino acids [18], which may favor the generation of hypoglycemic peptides, as DPP-IV and α-glucosidase inhibitory activities have been associated with low molecular peptides [16,17]. Exopeptidase activity also exerts a debittering effect, which helps to produce more palatable hydrolysates.

The other commonly used enzymes in the studies were Papain (EC 3.4.22.2) (n = 9; 7%); followed by Pancreatin (EC 232-468-9) or Corolase PP® (n = 10, 8%); Protamex®, Neutrase®, and Chymotrypsin (EC 3.4.21.1) (n = 6; 5% each); Bromelain, or Bromelin (EC 3.4.21.33) (n = 4; 3% each); Thermolysin® (EC 3.4.24.27), and Pronase E® (n = 2; 2% each). Protamex® (from *Bacillus licheniformis*, comprising a mixture of endo- and exopeptidases), Neutrase® (endopeptidases from *Bacillus amyloliquefaciens*), Thermolysin® (endopepti-dases from *Geobacillus stearothermophilus*), Pronase E® (from *Streptomyces griseus*, containing both exo- and endopeptidases) are microbial proteases [15,23-25] whereas pancreatin (from porcine pancreas), chymotrypsin (from bovine pancreas) [25,26], and Corolase PP® [27] are

digestive enzymes extracted from animal sources. Papain (papaya, Carica papaya), and bromelain (pineapple stem, Ananas comosus), in turn, are commercially available plant proteases [21,25]. Other proteases that appeared in just one study were alkaline proteinase, an animal proteolytic enzyme, PROTIN SD-NY10® (*Bacillus* metalloendopeptidase—EC 3.4.24.28), zingipain (ginger, *Zingiber officinale* EC 3.4.22.67), Protease from *Streptomyces griseus*, Proteinase K (EC 3.4.21.64), and Thermoase.

The proteolytic enzyme or set of enzymes used to obtain hydrolysates and peptides is possibly the single most important tool in the generation of bioactive peptides from food proteins. The peptides encrypted in the amino acid sequence of the proteins will be re-leased in their active form according to the specificity of the applied enzyme. Likewise, the ability to generate peptides that resist digestion or that generate active derivatives after the action of digestive enzymes also depends on the mode of action of the enzymes applied in the production of hydrolysates. Even considering only enzymes readily available on the market, at affordable prices, and in considerable quantities, the number of studies exploring the different possibilities of obtaining bioactive peptides in general and antidiabetic peptides, in particular, is still very small. There is a wide range of possibilities to be explored, including the systematic evaluation of the application of several different enzymes on the same protein substrate and the systematic study of the same enzyme used on several different protein sources.

3.4. Main Protein Substrate Sources

Peptides with bioactive functions can be obtained from various protein sources, and the most common are animal and plant dietary proteins. Protein substrates differ mainly in their amino acid sequences and their structural complexities. Likewise, bioactivity, environmental, social, and economic factors must be considered for generating hypo-glycemic peptides. For this study, among animal proteins, fish, chicken, pig, marine animals, and milk provided by different species of mammals were included. Among the proteins of plant origin, grains, oilseeds, and seeds of different vegetables and fruits were included. In addition to the conventional sources, non-conventional protein sources were also considered, as shown in Figure 4. These are unusual foods in the diet of most of the population so far, but they may be good sources of various nutrients, such as proteins and derived peptides.

Among the protein substrates for obtaining antidiabetic peptides, until 2020, according to Rivero-Pino and co-workers [18], the most studied were milk and soy proteins, owing to their high biological value when compared to other proteins. Of the selected articles, 37% (n = 28) used substrates of animal origin, of which cow's milk proteins (occurrence 9) were the most tested, not only for antidiabetic activity, but for several others as well. However, cows were not the only milk sources; camel's milk was also present (occurrence 7); as well as donkey's (occurrence 1) and mare's (occurrence 1). According to Akan [28] knowledge of the DPP-IV inhibitory activities of milk from non-bovine mammals is quite limited, and the α-glucosidase inhibitory activity from donkey milk is utterly unknown. Other animal protein sources were also studied: fish (occurrence 5), chicken (occurrence 2), pork (occurrence 1), and marine animals (occurrence 4), e.g., Antarctic krill, which is an important marine organism for sustaining the food chain in the Antarctic Ocean ecosystem as feed, and a source of all essential amino acids [29,30].

Plant protein sources were leaves, seeds, and fruits. Fruit seeds are considered an economical source of proteins and the limited knowledge about these proteins shows that this field still needs to be explored to elucidate questions about the potential bioactivity of these compounds both *in vitro* and *in vivo* [5]. Twenty-eight articles (37%) dealing with proteins of plant origin were selected, among which beans were the protein source that appeared in the largest number of articles (occurrence 5), followed by soybeans (occurrence 4) that, when compared to milk proteins, presented higher α-glucosidase inhibition capacity. Soy hydrolysates also presented the highest inhibition of DPP-IV when com-pared with other plant sources such as lupine and quinoa [13,19]. Common beans are widely consumed pulses all over the world owing to their high nutritional and nutraceutical values. Among pulses, common beans appear as a promising alternative to obtain bioactive naturally occurring, and also encrypted peptides with antidiabetic activity. The first report of potential peptides of common beans to inhibit DPP-IV and α-glucosidase enzymes appeared in 2016 and was proven in 2019 by *in vitro* and *in vivo* studies [31,32]. Other plant protein sources such as rice bran, quinoa, walnut, and wheat gluten had two occurrences each and, with one occurrence each, corn germ peptides, cumin seed, orange seed, perilla seed, rape napin, kiwicha, camellia seed, pigeon pea, and barley bran were selected. A study on oilseed

proteins and a comparison between vegetables (potatoes, sweet potatoes, yams, taro) were selected as well.

For the present survey, six articles from non-conventional protein sources were selected, among which four were about insects, one dealt with algae, and one was a review on peptides with DPP-IV inhibiting activity from marine organisms, including macro- and micro-algae, marine sponges, fish skin gelatin, and even tuna juice hydrolysates. In this work, the authors suggest that the valuable antidiabetic activities associated with bioactive peptides, derived from marine organisms and their metabolites, may be applied in the future by the nutraceutical and pharmaceutical industries with good results in the fight against diabetes [18,33]. Of the selected reviews, seven carried out studies with protein sources from several origins, three were focused on plant proteins, and one, as mentioned above, was based on marine sources.

Edible insects are among the unconventional food sources that, since January 1st, 2018, are considered novel foods by European countries (European Council Regulations No. 2015/2283, 2015). Insects have been gaining more and more space owing to the need to defocus from animal and plant protein sources, overloaded by environmental issues, and also because of their high protein content. Crickets, for instance, show approximately 62% protein (dry matter) in their composition [34]. Research with edible insect peptides is at an early stage, but with promising results so far. Given the lack of studies on DPP-IV inhibition activity, the comparison is difficult; however, the inhibition capacity of these hydrolysates ranged from 62–69%, especially with low molecular mass peptides (<1 kDa ~10 amino acids), which was considered a satisfactory rate by the authors, encouraging future *in vivo* tests [34,35]. In recent work with edible earthworms, Rivero-Pino (2020a) performed an ultrasound pre-treatment and managed to increase the percentage of α-glucosidase inhibition of the hydrolysate, opening up another research front for the preparation of new peptides.

As important as the choice of enzymes is the selection of the protein substrate for the production of antidiabetic peptides. This protein must be edible and non-toxic and must have peptides with antidiabetic activity encrypted in its amino acid sequence. The following characteristics are still desirable: low cost and low impact on the environment. An interesting alternative could be the use of proteins from agroindustry

residues and coproducts, which may help to lower residue generation, decrease waste, and contribute to the circular economy.

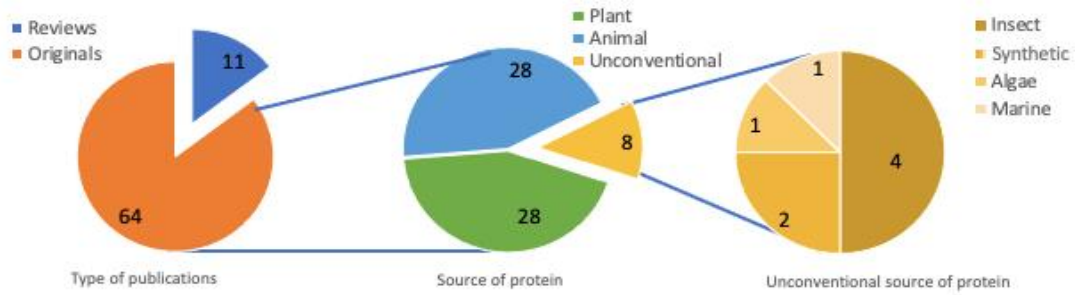

Figure 4. Source of the proteins used in the selected studies.

3.5. Bioactive Peptides with Multifunctional Activities

As shown in Figure 5, some of the published papers carried out studies that evaluated bioactivities other than antidiabetic capacity, focusing on the search for multi-functional peptides that act on various mechanisms of the consumers' welfare. The antioxidant and antihypertensive capacities were the most widely studied. Works that tested enzyme inhibition, such as α-amylase, α-glucosidase, or DPP-IV, to access only the hypoglycemic potential of peptides, representing 60% (n = 45) of all selected papers. Studies that evaluated two and three peptide functions, including antidiabetic activity, represented 21% (n = 16) and 15% (n = 11), respectively. The vast majority of the latter (10 out of 11 occurrences) tested antidiabetic, antioxidant, and antihypertensive capacity, whereas the other tested antidiabetic, antioxidant, and immunomodulatory capacity [34].

In several selected studies, multifunctional peptides were found; however, not all fractions exhibited dual or multiple bioactivities. Studies that performed peptide identification by mass spectrometry were able to identify multifunctional peptides more clearly, such as the case of the peptide KTYGL, from bean [36]; KVEPLP and PAL, from Antarctic krill; QHPHGLGALCAAPPST, from quinoa [37]; the di-peptides LN, VE, and IP also from bean. These are examples that presented the most potent simultaneous DPP-IV and ACE inhibition [15]. Free amino acids, from Atlantic salmon, such as Y, F, W, and P, also showed multifunction as ACE and DPP-IV inhibition, and

antioxidant activity [27]. In studies with synthetic peptides, Ibrahim et al. [38] identified that peptides STYV and STY were able to inhibit α-glucosidase and lipid accumulation in differentiated 3T3-L1 adipocytes.

Antioxidant activity was present in 19 works. This activity is usually attributed to low molecular weight peptides containing hydrophobic amino acids such as A, V, I, L, F, Y, P, M, and C, which increase their solubility in hydrophobic or lipidic phases. These hydrophobic amino acids are likely to interact with the S1 hydrophobic subsite of the DPP-IV active site [17,39,40] thus, they also act as hypoglycemic peptides. The presence of amino acids with hydroxyl groups (S, W, and Y) or basic amino acids (K and R) at the amino-terminus of peptides may play a critical role in α-glucosidase inhibition. Recent studies indicate that lower molecular mass peptides can also be responsible for the inhibitory activity of α-glucosidase, α-amylase, and DPP-IV [24,32,34,41]. In a study by Akan et al. [28], the correlation between the antioxidant activity by the ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) method and the inhibition of DPP-IV showed a coefficient of 0.97. The presence of hydrophobic peptides in the sequence may also be linked to the inhibition of the pancreatic lipase, delaying the di-gestion/absorption of triglycerides [37].

Seventeen works tested peptides for anti-hypertensive activity together with anti-diabetic activity. This activity is evaluated on the basis of the inhibition of the angiotensin I converting enzyme (ACE), which is a central component of the renin-angiotensin system, a potent vasoconstrictor that works by converting angiotensin I into angiotensin II, thus increasing blood pressure. Most of the purified DPP-IV inhibitory peptides reported were also di-, tri-, and oligopeptides. Dipeptides, such as IW, WL, and VY, naturally found in milk proteins and said to be antihypertensives, have also been identified in soy and rice proteins, showing that plant and animal proteins may be comparable sources of antidiabetic peptides. These smaller peptides can resist gastrointestinal digestion; thus, they may be absorbed into the circulatory system in an intact form [13,21]. The degree of hydrolysis plays an important role as most dietary protein derived peptides with ACE inhibitory abilities show relatively low molecular masses, generally ranging from di-peptides to pentapeptides, with molecular mass of 150–800 Da, and they contain positively charged K or R at the C-terminal end of sequences, which shows that the activity of a peptide may vary depending on the type of

amino acid and their position in the sequence. Peptides containing amino acids such as Y, F, W, and P may be strongly linked to ACE inhibition [21,27,34,40,41]. Other works dealing with multifunctions complementing antidiabetic activity were also selected; peptides from different sources were tested *in vivo* or *in silico*, as shown in Figure 5.

One of the most interesting features of bioactive peptides is their multifunctional potential. Thus, the same peptide may present several beneficial effects and, even if each individual effect is not very high, the combination of different bioactivities contributes to an overall desirable result.

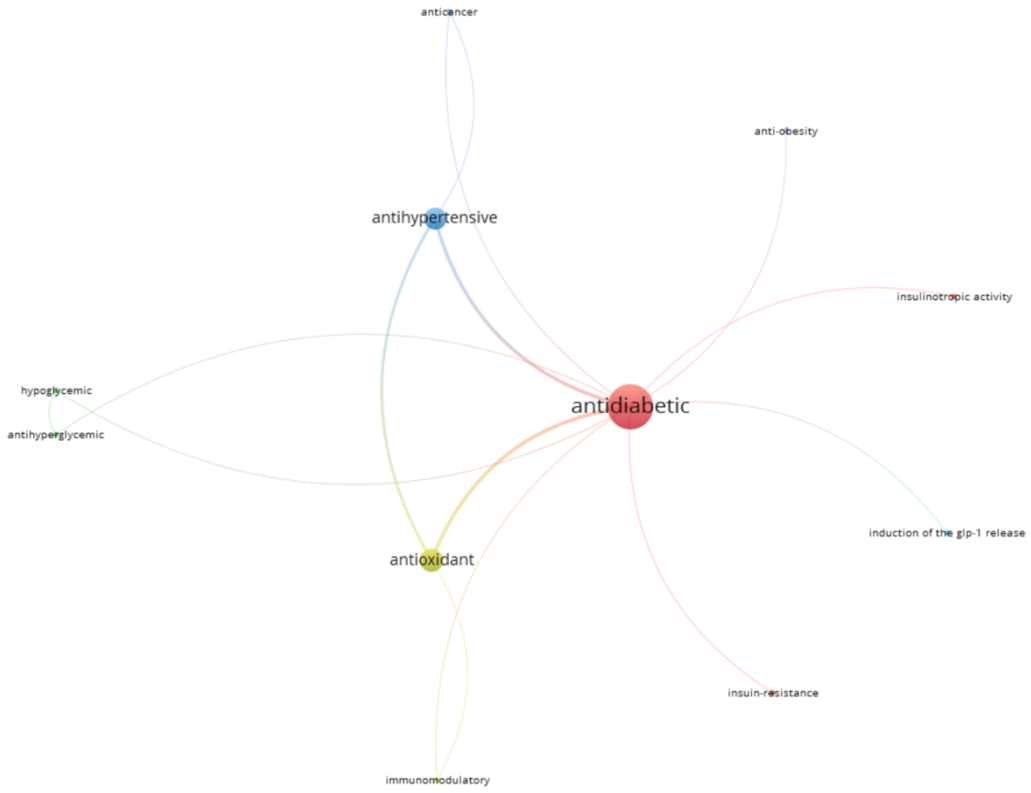

Figure 5. Network visualization map of the studied bioactive peptides with multifunctional activities. Node size is proportional to the number of occurrences (VOSviewer).

3.6. Type of Inhibition, Type of Analysis, and Type of Document

Table 1 shows that 32% of the articles (n = 24) were able to identify at least one sequence of peptides with a regular response function to diabetes, whether *in vitro* or *in silico*. This shows that omics and bioinformatics tools are extremely important to correctly identify and predict the probability of a given peptide to exhibit some

bioactivity, based on its amino acid composition and structure. The results for both, animal and plant peptides, as well as from other protein sources proved to be quite promising in this line of study, indicating good results even when compared to widely used synthetic inhibitors; the other articles are described in Table S1 (Supplementary data).

Authors (Year)	Source of Protein	Peptide Sequence	Outcomes of Interest
Mudgil, et al., [42]	Bovine milk (*Holstein Friesian*) and dromedary camel milk (*Camelus dromedarius*, local breed)	FLWPEYGAL; LPTGWLM, MFE and GPAHCLL and HLPGRG; QNVLPLH and PLMLP	Both potent inhibitory effects against enzymatic markers involved in diabetes, e.g., α-amylase, α-glucosidase and DPP-IV
Rivero-Pino, et al. [19]	Soy, Lupine, and Quinoa	EPAAV, NPLL, and APFTVV	Soy the most activity but chickpea, lentil, and pea also showed potent DPP-IV inhibitory activity.
Feng, et al. [43]	Camellia seed cake (*Camellia oleífera)*	SPGYYDGR, GLTSLDRYK, and GHSLESIK	Alcalase and Asp 542 was recognized as the key target amino acid of a-glucosidase.
Gao, et al. [44]	Bovine α-lactalbumin	ELKDLKGY and ILDKVGINY	These two peptides could bind with DPP-IV.
Ibrahim, et al. [38]	Synthetic peptides	STYV; STY; SEPA; SVPA	α-glucosidase inhibitory activity: STYV > STY > SEPA > SVPA; DPP-IV: SVPA; *In vitro* studies: SEPA.
Jia, et al. [45]	Whey protein	LDQWLCEK, VGINYWLAHK, LDQWLCEKL, KILDKVGINYWLAHK, ILDKVGINYWLAHK	The peptide LDQWLCEKL exhibited the highest inhibitory activity.
Jin, et al. [10]	Atlantic salmon (*Salmo salar*) skin	LDKVFR	Hydrolysate with MW < 3 kDa was an excellent source of DPP-IV inhibitory peptides.
Nongonierma, et al. [46]	Camel milk (*Camelius dromedaries*)	VPV, VPF, LPVPQ, YPI, and VL	The stability of VPV to gastric and intestinal digestive enzymes suggests that it may have potential as an antidiabetic agent for humans.
Vilcacundo, et al. [47]	Kiwicha (*Amaranthus caudatus*)	FLISCLL, SVFDEELS, and DFIILE	ACE, DPP-IV, and colon cancer cell viability were obtained. These digests also showed moderate α-amylase inhibitory activity.
Wang, et al. [48]	Soy protein	LLPLPVLK; SWLRL and WLRL	Development of novel antidiabetic peptide nutraceuticals with α-glucosidase, DPP-IV, and ACE inhibitory potential.
Xu, et al. [49]	Rapeseed (*Brassica napus*) napin	PAGPF, KTMPGP, IPQVS, and ELHQEEPL	
Zheng, et al. [50]	Casein-derived synthetic peptide	VPYPQ	VPYPQ was a promising casein-derived DPP-IV inhibitor.

Ibrahim, et al. [51]	Synthetic peptides	SVPA and SEPA	Two novel and active α-glucosidase inhibitory peptides were identified; they could resist GIT digestion and have the potential to retard postprandial hyperglycemia in diabetic patients.
Mune, et al. [15]	*Bambara bean*	IP, LN, VE, and VY	After simulated digestion, thermolysin showed significantly higher ACE and DPP-IV inhibitory properties compared to the Alcalase.
Nongonierma, et al. [39]	Camel whey protein (*Camelus dromedarius*)	FLQY, FQLGASPY, ILDKEGIDY, ILELA, LLQLEAIR, LPVP, LQALHQGQIV, MPVQA, and SPVVPF	LPVP and MPVQA, with DPP-IV inhibition, were identified for the first time in camel milk protein hydrolysates.
Ji, et al. [29]	Antarctic krill (*Euphausia superba)*	AP and IPA	Can be considered as a promising source of DPP-IV inhibitory peptides for use as natural food ingredients against type 2 diabetes.
Ji, et al. [30]	Antarctic krill (*Euphausia superba)*	LVGPLP and PAL	These peptides exhibited dual inhibition of ACE and DPP-IV.
Liu, et al. [52]	*Ruditapes philippinarum* hydrolysate	LAPSTM	*R. philippinarum*-derived peptides may have potential as functional food ingredients for prevention of diabetes.
Mojica, et al. [36]	Common bean *(Phaseolus vulgaris* L.)	KKSSG, KTYGL, GGGLHK, and CPGNK	The first report. Significant antioxidant, antidiabetic, and antihypertensive properties were found after gastrointestinal simulated digestion, and inhibition of DPP-IV and α-glucosidase.
Taga, et al. [53]	Wheat gluten	GPG, QPQ, QPF, LPQ, and SPQ	The novel gluten hydrolysate prepared using ginger protease can be used as functional food for patients with type 2 diabetes.
Uraipong and Zhao [41]	Rice bran (cultivar Reiziq)	GE, GG, GP, EK, and GH	*In vitro* simulated human gastrointestinal digestion led to substantial hydrolysis of these proteins, and the resultant peptides possessed significant -glucosidase and ACE inhibitory activities.
Vilcacundo, et al. [26]	Quinoa (*Chenopodium quinoa* Willd.)	IQAEGGLT, DKDYPK, and GEHGSDGNV	The peptides generated showed ability to inhibit enzymes involved in incretin degradation and digestion of dietary carbohydrates.
Lammi, et al. [54]	Soy and Lupin Protein	Soy 1 (IAVPTGVA) and Lup 1 (LTFPGSAED)	Soy 1 (IAVPTGVA), Soy 2 (YVVNPDNDEN), Soy 3 (YVVNPDNNEN), Lup 1 (LTFPGSAED), Lup 2 (LILPKHSDAD), and Lup 3 (GQEQSHQDEGVIVR), were screened for their capacity to inhibit the activity of DPP-IV, using an *in vitro* bioassay against human recombinant DPP-IV.
Nongonierma, et al. [55]	Bovine α-lactalbumin	GY, GL, GI, NY, and WL	This preliminary study demonstrated the benefit of using a targeted approach combined with an experimental design for generation of dietary protein hydrolysates with DPP-IV inhibitory properties.

In silico analyses using bioinformatics tools totaled seven articles (9%) in the 2019-2021 range. On the other hand, *in vitro* studies totaled about 42% (n = 32) in the same period. Regarding the *in vivo* analysis, only three articles (4%) performed tests in rats, two with peptides of plant origin (hydrolyzed common beans and walnut peptides) [32,56], and the other with peptides of animal origin (hydrolyzed chicken feet) [57]. Rivero-Pino et al. and Acquah et al., both in 2020, reviewed the production and functionality of peptides that were tested *in vivo*.

Table S2 (Supplementary data) shows the type of inhibition, type of analysis, type of document, and citation count of the 75 selected articles. Most of the articles (n = 39, 53%) tested the ability of the peptides to inhibit just DPP-IV, followed by articles that tested the inhibition against all antidiabetic-related enzymes—α-glucosidase, α-amylase, DPP-IV (n = 11, 15%). Two review articles did not provide information about the study inhibitions. In general, the most tested inhibition capacity was against DPP-IV (n = 59), followed by inhibition against α-glucosidase (n = 31) and α-amylase (n = 23).

DPP-IV is a metabolic enzyme that is expressed in several tissues, such as kidney, liver, and intestinal brush-border membrane, which cleaves dipeptides from the N-terminus of polypeptides, in which proline or alanine is at the penultimate position (Taga et al., 2017). This enzyme regulates the degradation of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) incretin hormones according to physiological needs [11,58]. GLP-1 and GIP are hormonal peptides released after the ingestion of food, which regulate blood glucose levels by stimulating insulin secretion from the pancreas in a glucose-dependent manner and inhibit glucagon release by pancreatic cells [53,59]. However, DPP-IV quickly degrades them, leading to a very short half-life of those incretin hormones. Thus, DPP-IV inhibitors can extend GLP-1 and GIP action, and also enhance blood glucose regulation, which is considered a novel treatment against T2D [58]. DPP-IV activity has been related to metabolic syndrome (MS) in obese children and adolescents by El-Alameey et al. [60], where serum DPP-IV activity of obese patients with MS was significantly elevated in relation to obese patients without MS and control subjects. Trzaskalski et al. [61] also attributed to DDP-IV an important role in metabolic disease. In this instance, however, the authors indicated a direct protein–protein interaction between DPP-IV and different ligands such as adenosine deaminase (ADA), caveolin-1,35 extracellular matrix (collagen and

fibronectin), and C-X-C chemokine receptor 4 (CXCR4) as responsible for the inflammation process both in obesity and T2D. In this case, the simple inhibition of the catalytic activity of the enzyme would not prevent the installation or progression of MS.

Alpha-glucosidase is a membrane-bound enzyme present in the epithelial mucosa of the small intestine (brush border of the enterocytes). This enzyme hydrolyses 1,4-α-glycosidic linkages present in oligosaccharides and then converts these long-chain carbohydrates into monosaccharides that are absorbable from the intestine into the blood. By inhibiting α-glucosidase, the digestion of complex carbohydrates is retarded; there-fore, the overall absorption of glucose in the blood is delayed and hyperglycemia is prevented [59]. Alpha-amylase hydrolyzes complex carbohydrates, such as starch, into oligosaccharides, which may be further hydrolyzed by α-glucosidase. This enzyme is secreted by the salivary and pancreatic glands [18]. The inhibition of α-amylase may lead to an overall decrease in blood glucose levels, as this enzyme is among the leading enzymes of carbohydrate digestion in the body [59].

Eighty-five percent of the articles in this survey were original articles (n = 64) and the other fifteen percent were review articles (n = 11). Most of the 75 selected articles per-formed *in vitro* studies, only two articles did not mention the type of analysis performed, which were review studies. Of the 64 original works, 42 performed only *in vitro* assays, 15 tested both, in vitro and *in silico*, 2 *in vitro* and *in vivo*, 1 *in vitro* and *in situ*, and 1 *in vitro*, *in vivo*, and *in situ*, and 3 performed only *in silico* studies. Among the review articles, most provided an overview of studies that reported *in vitro* and *in vivo* (n = 4); *in silico* and *in vivo* (n = 2); *in vitro* and *in silico* (n = 1); and *in vitro* (n = 1) anti-diabetic studies and one review paper included all three types of antidiabetic evaluations. These reviews discussed antidiabetic effects of protein hydrolysates and peptides generated from cereals and other types of grains, marine organisms, plants, and multiple protein sources. In addition, they showed recent advances in the subjects of how antidiabetic peptides are produced, as well as their properties, and the challenges of testing bioactive antidiabetic peptides *in vivo*.

The most cited articles were by authors Vilcacundo et al. [26], with 67 citations; followed by Uraipong and Zhao [62], with 61 citations; and Mudgil et al. [63], with 57 citations. These are original articles with *in vitro* tests. Vilcacundo et al. [26] evaluated quinoa protein, a plant protein, as well as Uraipong and Zhao [62], who

studied rice bran proteins, while Mudgil et al. [63] tested hydrolysates of animal origin: camel milk proteins. The first two studies evaluated the multifunction of the peptides, such as antidiabetic and antiobesity; and antidiabetic and antihypertensive, respectively, while the last one evaluated only antidiabetic inhibition. As for the outcomes, the three studies concluded that the peptides generated from enzymatic hydrolysis showed the potential to be used as functional foods or for nutraceutical applications for the control of diabetes.

In most recent investigations, the inhibitory activities of enzymes by peptides are generally evaluated by *in silico* or *in vitro* assays, and most authors agree that *in vivo* experiments involving activity assays are needed to demonstrate the physiological effect of the peptides. However, the evaluation of the *in vivo* inhibitory activity of peptides is recommended to be performed after screening for inhibitory activity *in vitro*, as the in vivo analyses require longer times to study the effects in animals, usually between 4 to 6 weeks, in contrast to *in vitro* enzyme inhibition tests, which take only a few hours. Of the selected articles, eight mention *in vivo* analyses, five of them are reviews on antidiabetic peptides, two with *in vivo* analyzes using laboratory rats [32,57], and one with *in vitro* analyzes on cells and also *in vivo* analyses on rats [56].

According to Aquac et al. [1], the inhibition pathways studied so far used by the peptides are: inhibition of carbohydrases such as alpha-amylases and glycosidases; stimulation of incretin hormone production; stimulation of hormones that control satiety and gastrointestinal emptying and inhibition of DPP-IV. Recent studies involving the regulatory mechanisms of glucose metabolism by protein hydrolysates and peptides in general are relatively limited. There is a lack of scientific evidence on the molecular mechanisms directly related to the regulation of glucose metabolism at the cellular level. Studies have reported that proteins and peptides can regulate glucose metabolism by inhibiting DPP-IV activity in Caco-2 cells, stimulating GLP-1 secretion in GLUTag cells, and promoting glucose uptake in insulin-resistant HepG2 cells [6].

Wang et al. [56] studied the *in vivo* inhibitory activity of walnut hydrolysates with molecular weight of 3–10 kDa due to their higher alpha-glucosidase inhibitory activity (61.73%) and their ability to facilitate glucose uptake in insulin resistant HepG2 cells *in vitro*; and using rats with streptozotocin-induced diabetes (STZ). After six weeks of oral administration of the hydrolysates, the peptides alleviated insulin

resistance by increasing its secretion and the levels of glucokinase and glycogen in the liver, as well as decreasing the blood glucose level in fasting of diabetic rats.

The same effect of decreasing glucose levels was observed in a study with peptides obtained from beans, which were administered orally to normoglycemic fasting rats and measured by starch tolerance tests after 45 min of starch ingestion [32]. Casanova-Marti et al. [59] showed that peptides from chicken feet reduced blood glucose in glucose intolerant rats, which may be partially due to their DPP-IV inhibitory activity, and also stimulated endogenous GLP-1 secretion, possibly involved in the antihyperglycemic action of this hydrolysate.

4. Challenges and Perspectives

The interest in studying peptides with bioactive potential has been growing in recent years, and this field has become very promising in the food and nutrition sciences. With the advance in science and technology, more studies are emerging from several protein sources, leading to the discovery of novel peptides, which may be used as natural food-derived peptides remedies, avoiding the usual side effects of the existing drugs. Thus, researchers have been exploring the potential of bioactive peptides as a new class of therapeutic drugs.

To archive success and acceptability when using a therapeutic agent, its delivery needs to be evaluated. The main form of drug administration is the oral route; thus, an oral active therapeutic agent must be stable in the gastric microenvironment. Even though peptide-based drugs are specific to their target and effective, oral bioavailability is still an issue that restricts the acceptance of this kind of drug and its market value [5].

As challenges, some of the articles addressed the investigation of the stability of peptide-based drugs under simulated gastric and intestinal conditions, as well as the bioavailability, metabolism, excretion, stability, and toxicity profile of the peptides. Owing to the extensive hydrolysis of peptides in the gastrointestinal tract by peptidases in the stomach, small intestinal brush border, and low cellular uptake of these peptides, the hydrolyzed peptides usually present very low oral bioavailability [64].

Most studies in this paper predict, as the next steps in research with antidiabetic peptides, the application of *in vivo* assays to unravel their mechanisms of action in live models, using animal models and human tests; research on the structure of the peptides to confirm their sequences, in different ways, such as isolation and sequencing; investigation of the relationship between the structural characteristics of peptides and their hypoglycemic activities; and the investigation of optimal strategies to scale-up the pro-duction, as well as the industrial optimization of the enzymatic production of bioactive antidiabetic peptides. Many are said to hold promise for replacing drugs in the future or serving as efficient functional food in preventing diabetes. For the case of peptides that were described for the first time in the last five years, more *in vitro*, *in vivo* studies, and cell culture assays are also needed to validate and confirm their functionality, and to provide a higher level of evidence.

5. Conclusions

A bibliometric analysis was carried out to identify research on antidiabetic bioactive peptides to be used as food ingredients and nutraceuticals. From what could be found in the available literature of the last 5 years, several studies have been conducted from different sources of food proteins, novel or traditional, with different commercial enzymes in order to obtain antidiabetic peptides. However, the compilations of these works still do not allow us to truly determine which types of proteins, of enzymes, and under which conditions the best peptides, or the best yield of peptides, with that specific function, are obtained. It can therefore be concluded that a more systematic investigation, possibly with the aid of bioinformatics tools, is desirable to help unravel the best approach for further upscaling of antidiabetic peptides, or any kind of bioactive peptides, production from dietary proteins. It is hoped that the compilation of findings in this paper will help re-searchers in this field to optimize strategies to point out for the best means of production of antidiabetic bioactive peptides to help improve the management and control of diabetes worldwide.

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CAPÍTULO III

BIOACTIVE PROPERTIES OF PEPTIDE FRACTIONS FROM BRAZILIAN SOY PROTEIN HYDROLYSATES: *IN SILICO* EVALUATION AND EXPERIMENTAL EVIDENCE

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ABSTRACT

Soybeans are a known source of dietary proteins and potential bioactive peptides. In this study, a protein hydrolysate from soybean protein concentrate was produced using papain. The peptides were separated by ultrafiltration (< and > 3 kDa, LMMH (low molecular mass) and HMMH (high molecular mass), respectively) and sequenced through LC-MS/MS. To obtain a thorough identification of the peptides in the hydrolysate, different analysis methods and bioinformatics techniques were applied, covering a molecular mass range from more than 480 Da up to small dipeptides. The antioxidant and the inhibitory α-glucosidase and lipase potentials were evaluated by different *in vitro* tests. Sixty-nine peptides were identified in the HMMH fraction and 32 in LMMH, but only 16 matched the 118 sequences obtained by *in silico* simulated hydrolysis. Unlike previous reports, the HMMH fraction showed higher antioxidant activity, by all 5 *in vitro* methods applied, which was not accompanied by the *in silico* evaluation. Both high and low molecular mass fractions showed similar inhibitory activities against α-glucosidase and pancreatic lipase. LMMH, however, showed better results for α-glucosidase inhibition (IC50 = 0,94), in agreement with the *in silico* evaluation. This combination of bioactivities makes the fractions of this hydrolysate potential food ingredients with the possible ability to delay the lipid peroxidation of meat products, limiting the digestion of lipids in the product and also with the potential to delay the digestion of carbohydrates ingested in the same meal.

Keywords: antioxidant; antidiabetic; antiobesity; mass spectrometry; multifunctional peptides

1. INTRODUCTION

Soybean (*Glycine max*) is the most economically important legume in the world, providing dietary protein to millions of people and ingredients for hundreds of products. Currently, there are numerous commercial products that are based on soy or contain soy ingredients (Yi et al., 2020). Soy is an abundant and relatively inexpensive source of protein with high nutritional value and excellent functional properties (Singh and Hati, 2014). Different studies have been carried out to identify the bioactivities of soybean peptides such as antioxidant (Coscueta, et al., 2016; Yi et al., 2020; Lammi et al., 2019), antitumor (Rayaprolu et al, 2017; Gonzalez-Montoya et al., 2017; Chen et al., 2018; Hao et al., 2019), antihypertensive (Lee and Hur, 2017; Wang et al., 2019; Daliri et al., 2019) and antidiabetic (Rivero-Pino et al., 2021; Wang et al., 2019; Jahandideh et al., 2022;) activities, among others.

Proteins in foods may exert biological activities that promote health, in addition to their expected nutritional value. Food proteins are among the precursors of many biologically active peptides that may be released during proteolysis (gastrointestinal digestion, *in vitro* hydrolysis) or during food processing (cooking, fermentation, ripening). Bioactive peptides, when administered orally as part of the diet, might affect the body's major systems (Daliri et. al., 2018).

Antioxidant peptides may act in the preservation of food products presenting significant advantages, such as easy absorption and little to no harmful effects. These peptides may reduce deterioration and discoloration as a result of the oxidation of fats, interrupting peroxidation in the initiation or propagation phases (Huang et al., 2017). The use of synthetic antioxidants

frequently applied in the food industry has been restricted because of their potential risk to the human health. Hence the growing search for natural antioxidants that function as natural additives has become quite significant (Sanjukta and Rai, 2016).

Peptides that inhibit carbohydrate digestion include α-amylase and α-glucosidase inhibitory peptides. ∝-glucosidase inhibitors may limit carbohydrate absorption in the intestine and control blood glucose. This activity involves the competitive inhibition of α-glucosidase in the small intestine, reducing the rate of starch decomposition into glucose, thereby slowing glucose absorption in the intestine and reducing postprandial hyperglycemia (Yan et al., 2019). Peptides of plant and animal origin have already presented the ability to inhibit these enzymes, showing a very promising path to be followed (Feng et a., 2021; Han et al., 2021; Megrous et al., 2020).

Obesity is among the risk factors associated with type-2 diabetes, which ranges from the absence of symptoms to complications with enormous risks of morbidity and mortality. A plausible pharmacological target for controlling obesity is to reduce the digestion and absorption of dietary fat in the digestive tract. Lipases are enzymes that catalyze the breaking of the ester bond in triglycerides to form monoglycerides and free fatty acids, the more absorbable forms, which will then be stored in adipose tissues, leading to obesity. Inhibition of pancreatic lipase slows down this process, which may be a viable approach to the control of hyperlipidemia and obesity (Velasquez et al., 2007; Younis et al., 2021; Alnuaimi et al., 2023).

A key challenge in bioactive peptide development for therapeutic or industrial purposes has been the difficulty in establishing a cause-and-effect

relationship between bioactive peptide consumption or application and their intended health effects in humans or functional effects in foods (Daliri, et al, 2018). The bioinformatic (*in silico*) approach relies on information available on databases to determine the occurrence of already identified bioactive peptides in a protein or hydrolysate of interest. The theoretical predictions are considered useful tools in the discovery of peptide bioactivities (Minkiewicz et al., 2019; Zhao et al., 2019).

The purpose of the present study was to produce soy protein concentrate hydrolysates applying papain, an enzyme that, for some reason, is not so widely studied as other readily available proteases for the production of bioactive peptides (Tacias-Pascacio et al., 2021), to analyze them by "shotgun" peptidomics and to match the sequences with databases of antioxidant activity and anti-hyperglycemic activity, in order to pinpoint those most likely to impart these bioactivities. To validate the predicted results, the antioxidant capacity of the fractions was evaluated by five different methods and the *in vitro* inhibition of α-glucosidase and pancreatic lipase was assayed to estimate the possibility of glycemic and lipid metabolism control, respectively.

2. MATERIAL AND METHODS

2.1 Plant, enzyme and food material

Soy protein concentrate (SPC) containing 75% of total protein was obtained in the local market. The purified proteolytic enzyme (papain EC 3.4.22.2) used was purchased from Sigma® (3 U/mg). Papain was selected due to its relatively low market price, easy obtainment and because its plant/vegan status. A lot of sardines was purchased at a local fair and was stored at -18 °C

until use. The enzyme:protein (E:P) ratio was set at 1:80, according to previous tests (data not shown).

2.2 Hydrolysis of soybean protein concentrate

Hydrolysis was conducted based on the modified method by Contreras et al. (2011). Briefly, samples of 2.7 g of SPC were added to 100 mL of phosphate buffer (50 mM; pH 7.0) and heated at 95 °C, for 15 min then cooled and kept in a bath at 60 °C. The addition of protease initiated the reaction After 30 minutes of reaction, the medium was heated at 100°C/10 min. cooled and centrifuged at 13,000 x *g* for 15 min. (Thermo Fisher Scientific, USA). The supernatant was aliquoted and freeze dried. A not hydrolyzed sample (NH), where no enzyme was added, was used as control.

2.3 Degree of hydrolysis (DH%)

The DH% was determined according to Castro and Sato (2014). The total protein content was analyzed by the method of Lowry (1951), applying bovine serum albumin (BSA) as standard. For the peptide content evaluation, 1 mL of the hydrolysate was added to an equal volume of a 0.44 mol.L^{-1} TCA solution. The mixture was incubated for 30 minutes at room temperature and then centrifuged at 13,000 x *g* for 15 min. The supernatant was analyzed by the same method and the results were expressed according to Equation [1].

$$DH\% = [soluble \in 0.22M\ TCA\ peptides]/[Total\ protein] * 100 \quad [1]$$

2.4 Ultrafiltration

After hydrolysis, the total hydrolysate (TH) was separated by ultrafiltration process applying centrifuge devices (Amicon Ultra-4 Filters with Ultracel-3 membrane, Millipore, USA). Thus, fractions of the hydrolysate containing peptides of molecular masses (MM) of < 3 kDa (LMMH - low molecular mass hydrolysate) and > 3 kDa (HMMH - high molecular mass hydrolysate) were obtained. The samples produced were freeze dried and stored at -18ºC until use.

2.5 Peptides identification

2.5.1 Protein digestion and analysis by liquid chromatography-mass spectrometry (LC-MS/MS)

The LMMH was filtered and directly subjected to liquid nano-chromatography and mass spectrometry analysis. The HMMH was precipitated with TCA (15% v/v) for 2 h at 4 °C, washed twice with cold acetone and centrifuged at 13,000 x *g* for 15 min at 4 °C. The precipitate was resuspended in triethylammonium bicarbonate buffer (TEAB) (50 mM; pH 8.5). For digestion, 100 µg of protein were reduced by the addition of 7 mM dithiothreitol at 60°C for 30 min and alkylated with 14 mM iodoacetamide in 50 mM TEAB for 30 min at 25°C. Trypsin (Promega) was added, in a E:P ratio of 1:50 (w:w), and incubated for 16 h, at 37°C. After digestion, samples were acidified with 1% formic acid, centrifuged at 13,000 x *g* for 15 min and filtered. Protein quantification of all samples was obtained by fluorimetry using the Qubit system (Qubit® quantitative fluoride - Invitrogen).

Each filtered sample was analyzed in a technical triplicate. The nano-chromatography system used was the Easy-nLC 1000 (Thermo Fischer Scientific). The nLC-MS/MS was performed according to Amorim et al. (2017) at

Mass Spectrometry Facility - RPT02H, Carlos Chagas Institute, Fiocruz, Curitiba, Parana, Brazil.

LMMH was also evaluated for the presence of di- and tripeptides as follows: 0.1 g of the LMMH were dissolved in 1 mL of 50 mM ammonium bicarbonate solution in water. This was then vortexed for 40 s, centrifuged for 15 min, then the supernatant was collected. The dilution was carried out with 100 µL of the supernatant and mixed with 900 µL of the mobile phase, vortexed for 1 min, centrifuged at 4,000 rpm for 10 min and left at −20°C for 1 h. In the second dilution, 100 µL of supernatant was diluted in 900 µL of the mobile phase. Diluted samples were directly injected into the LC–MS/MS system.

The mobile phase was pumped isocratically using a mixture of acetonitrile:water:formic acid (70.0:29.9:0.1 v/v/v) at a flow rate of 300 µL min^{-1} set on LC pump Waters 515, with an XBridge (Waters) C18 3.5 µm (4.6 × 50 mm) column. The volume injection of the samples was 5 µL using the mass spectrometer rheodyne valve, running for 5 min at room temperature. MS and MS/MS experiments were conducted on a PremierXE triple-quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA) using a conventional electrospray (ESI) ionization source operating in the positive ion mode. Desolvation gas and source block temperatures were 350°C and 110°C, respectively. The ESI source was operated in the positive ion mode (ESI+) at 4.0 kV. The cone voltage, collision energy, and collision gas pressure (Argon) were 20 V, 15 V, and 3.0 × 10^{-3} Torr, respectively (all parameters were optimized previously) (Poliseli et al., 2021).

2.5.2 Peptides Sequencing

For the identification of the peptides of the LMMH fraction, initially, two complementary approaches were applied: (1) match to a “soybean” database and (2) *de novo* sequencing. For the first approach two different softwares were used: the Comet software of the PatternLab platform (Carvalho, Lima et al., 2016) and the Peaks software (Zhang, Xin et al., 2012). The latter was also used for the *de novo* sequencing. For the searches made against the database, peptides with FDR 2%, delta mass of 5 ppm and Min Pri Score of 2.50 were accepted. For the *de novo* sequencing strategy, candidates with % confidence > 70 and error < 20 ppm were accepted.

Di- and tripeptides candidates were identified using neutral loss scan of 46 Da for formic acid loss of carboxylic acid. The first screening was comparing to the mass match to di- and tripeptides using 20 possible amino acids. The candidates were submitted to collision induced dissociation experiment, and the y1 ions for amino acids were evaluated together to the amino acid residue, immonium and related ions for the manual *de novo* sequencing (Poliseli et al., 2021).

For the HMMH fraction, the .RAW files were analyzed with a Peptide-Spectrum Match (PSM) strategy using the Comet PSM program from the PatternLab platform (Carvalho, Lima et al. 2016) and a database containing 261,019 entries for "soybean", obtained from the UNIPROT bank (http://www.uniprot.org/). The accepted false discovery rate for peptides and proteins was less than or equal to 1% FDR, using a target-decoy reverse database, containing sequences of soybean proteins, reverse sequences and common contaminants, available at the "PatternLab's Search Database Generator". Peptides with up to three missed cleavages, oxidation of

methionine (+15.9949 Da) as variable modification; 40 ppm of mass tolerance for the precursors and 1.005 Da of mass tolerance for the fragment ions were accepted. For the theoretical reconstruction of the peptides in the HMMH fraction, the proteins identified in the soybean database were digested *in silico* with papain using the mMass program (Niedermeyer and Strohalm, 2012). Up to 5 missed cleavages were accepted. A search was then made against the *in silico* peptide library in order to locate the original peptides that generated the identified peptides, after trypsinization, considering the existing peptides and the specificity of trypsin (cleavages after K or R).

All identified peptides were validated using the Search Engine Processor (SEPro), also from the PatternLab platform. A value of 5 residues was established as the minimum length of the identified peptides and sequences with less than 5 ppm of error were accepted.

2.5.3. Remapping of *in vitro* papain digested peptides to potential pertaining proteins through *in silico* hydrolysis and alignment workflow

Main *Glycine max* storage proteins sequence data were processed through the RapidPeptidesGenerator package (RPG, Maillet et al., 2020, https://rapid-peptide-generator.readthedocs.io/en/latest/index.html), by implementing the command line “rpg -i input_file.fasta -o output_file.fasta -e 32”. The “-i” flag and the “input_file” command refers to the respective .fasta file of the protein to be digested; the “-o” flag and the “output_file” command refers to the file generated after *in silico* digestion; the “-e” flag and the “32” command refers to the papain protein from the RPG database (Maillet et al., 2020). The proteins’.fasta files were obtained from the UniProt platform (https://www.uniprot.org, Release 2022_02). Digested proteins and their UniProt identifiers were: glycinin G1 (P04776), glycinin G2 (P04405), glycinin G3

(P11828), glycinin G4 (P02858), glycinin G5 (P04347), beta-conglycinin beta subunit 1 (P25974), beta-conglycinin alpha' subunit (P11827), beta-conglycinin alpha subunit 1 (P0DO16), beta-conglycinin beta subunit 2 (F7J077), beta-conglycinin alpha subunit 2 (P0DO15), beta-conglycinin beta subunit (Q50JD8). According to the RPG package documentation (https://rapid-peptide-generator.readthedocs.io/en/latest/enzymes.html#pap), “papain preferentially cleaves after R or K (P1) preceded by A, V, I, L, F, Y or W in P2. It won't cleave if followed by V in P1'”. The definitions for cleaving rule are (A or V or I or L or F or Y or W) (R or K,) and for exception rule (A or V or I or L or F or Y or W) (R or K,) (V). The files were reformatted, removing the .fasta file headers, leaving only the digested peptide sequences to be processed in the next step.

In order to establish a baseline for peptides comparison of *in silico* digested sequences with data acquired from mass spectrometry (MS) experiment, the find-pep-seq tool was created in Python language by our workgroup (https://github.com/joanapaulaso/find-pep-seq/). The sequences, both computational and experimental, were transformed into vectors representing the number of times each unique amino acid was repeated among paired sequences. Based on the result, the cosine similarity (Li and Han, 2013) value was calculated. Paired peptide sequences with a difference of more than 8 amino acids in total were disregarded. The cosine similarity score of 0.90 was established as the cutline. The *in silico* and MS sequences preprocessed by find-pep-seq were, then, processed by the online tool SIM - Alignment Tool for protein sequences (Expasy, https://web.expasy.org/sim/), based on local similarity algorithm (Huang and Miller, 1991), in which paired sequences were scored based on the successful alignment of their amino acids. The results

were organized and the differences between the sequences were highlighted (Supplementary table 1). By applying this workflow, peptide sequences obtained after *in vitro* digestion with papain were remapped to the potential storage proteins to which they respectively pertained.

2.5.4 In silico bioactivity search for the identified peptides

MS acquired sequences that were successfully remapped to potential storage proteins were processed by the online BIOPEP-UWM tool (Minkiewicz et al., 2019, https://biochemia.uwm.edu.pl/en/biopep-uwm-2/) to assess if the *in vitro* tested activities were related to them through an *in silico* approach. None of the sequences had related pancreatic lipase inhibitor activities, thus, only antioxidative activity and α-glucosidase inhibition were considered. Subsequently, these sequences were processed through the online PeptideRanker tool (Mooney et al., 2012, http://distilldeep.ucd.ie/PeptideRanker/), in order to rank peptides based on their bioactivity potential. PeptideRanker calculated scores (A%) are listed within Supplementary table 1.

The biological activity index (A%) of each sequence was calculated from the ratio between the sum of the fragments that showed activity and the total number of amino acids in the respective sequence (Iwaniak et al., 2019). Data acquired from the proteomics experiment were assessed to elucidate the ratio in which each protein type (glycinin or conglycinin) was expressed. Calculation was based on the mean value of normalized spectral abundance factor (NSAF) by storage type, resulting in 37% of glycinin and 63% of conglycinin. Such proportions were applied as a weighting factor of the bioactivity potential

indicated by the PeptideRanker scores (A%), by which they were accordingly multiplied.

Values of A% and bioactivity indexes resulted from *in vitro* analysis (antioxidative activity and α-glucosidase inhibition for NH, LMMH and HMMH samples) were normalized through the min-max method (Sheng et al., 2019) as presented in Equation 2, within each sample group defined by bioactivity. Standard error of the mean was calculated for each group. To clarify the statistic significant differences among samples, ANOVA and post-hoc Tukey HSD test were applied using RStudio software (RStudio 2022.02.3).

$$x' = \frac{x - \min(X)}{\max(X) - \min(X)} (max_{new} - min_{new}) + min_{new} \quad [2]$$

in which x refers to the unnormalized value from the *in vitro* bioactivity assessment index value; min(x) and max(x) refer to the minimum and maximum unnormalized bioactivity assessment index values; min_{new} and max_{new} refer to the minimum and maximum values from calculated A%; and x' refers to the resulted normalized A% value.

2.6 Antioxidant activity

The antioxidant activity of the fractions was assayed by four different methods as a means of elucidating the mode of action and/or some of the characteristics of the peptides in the samples.

The DPPH method was assayed according to Rufino et al. (2007); in methanol, at 515 nm, after a 60 min reaction; the ABTS method was carried out according to Re et al. (1999), in ethanol, at 734 nm, after a 6 min reaction; the FRAP method was applied according to Benzie and Strain (1999); at 595 nm,

after a 30 min reaction, at 37ºC. All readings were performed using a Shimazdu UV-200 (Japan) UV-Vis spectrophotometer. The ORAC procedure was performed as the methodology by Huang et al. (2002), with fluorescein, after a 3-hour reaction, at 37ºC and fluorescence conditions of excitation at 485 nm and emission at 535 nm, applying a micro-plate reader (Spectramax i3x, Molecular Devices). For all determinations, a standard curve of Trolox was prepared and all results were expressed as TEAC (Trolox Equivalent Antioxidant Capacity).

2.7 Antioxidant activity in food model

2.7.1 Preparation of sardine samples

The preparation of the homogenates was as follows: from each sardine, the head, caudal fin and viscera were removed with the aid of a knife and the remaining material was washed in running water. Subsequently, the sardines were thoroughly homogenized in a food processor and divided into 6 trials, with 40 g of sardines for each trial. The sardine samples were added with antioxidant, when appropriate (standard butylated hydroxytoluene - BHT or hydrolysate sample), and then stored in transparent polyethylene bags according to the conditions described in Table 1, in triplicates.

Table 1. Test conditions in food model

Tests	Amount of antioxidant	Temperature
Refrigerated blank	0 mg	8 ºC ± 2º C
Frozen blank	0 mg	-12 ºC ± 2º C
Sardine + HMMH[1]	40 mg	8 ºC ± 2º C
Sardine + LMMH[2]	40 mg	8 ºC ± 2º C
Sardine + NH[3]	40 mg	8 ºC ± 2º C
Sardine + BHT[4]	40 mg	8 ºC ± 2º C

[1]High molecular mass hydrolysate; [2]Low molecular mass hydrolysate; [3]non-hydrolysed; [4]butylated hydroxytoluene

2.7.2 Index of Thiobarbituric Acid Reactive Substances (TBARS)

The value of the thiobarbituric acid reactive substance index (TBARS) was determined in the sardine samples, according to the method of Vyncke (1970), with small modifications, to evaluate the oxidative stability during the storage period, as applied by (Coelho et al., 2019). The results were expressed as mg of malonaldehyde/kg homogenate. The analysis of TBARS was carried out over five days of storage at the following points: T0, T1, T2, T3 and T4, with a 24-hour interval between each point, and T0 samples were analyzed immediately after the preparation of each sample group.

2.8 Enzymatic inhibition

2.8.1 α-Glucosidase inhibition assay

The α-glucosidase inhibitory activity was evaluated according to Ibrahim et al. (2018), with slight modifications. Samples, diluted in water (50 µL) at final concentrations of 8 to 12 mg. mL^{-1}, or ultrapure water (control) were incubated with 25 µL of 0.5 $U.mL^{-1}$ α-glucosidase solution (EC.3.2.1.20) (G5003- Sigma Aldrich®) in PBS (100 mM, pH 6.8), at 37°C, for 1 h. After pre-incubation, 25 µL of 4-nitrophenyl α-D-glucopyranoside (p-NPG) substrate solution (5 mM) in PBS (100 mM, pH 6.8) was added, and the mixture was incubated at 37°C, for 30 min. To stop the reaction, 100uL of glycine-NaOH buffer (pH 10.0) were added. The absorbance was measured at 405 nm in a microplate reader. The results were expressed as IC50 values (concentration that inhibits DPP-IV activity by 50%, n = 3).

2.8.2 Pancreatic lipase inhibition assay

The pancreatic lipase inhibitory activity was evaluated based on the method described by McDougall et al. (2009), adapted to a 96-well microplate. Samples (15 µL) at final concentrations of 8 to 12 $mg.mL^{-1}$ or ultrapure water (control) were incubated with 60 µL of pancreatic lipase from porcine pancreas (EC 232-619-9) (L0382 - Sigma Aldrich®) (10 $mg.mL^{-1}$) solution in Tris-HCl buffer (100 Mm, pH 8.2), at 37°C, for 30 min. After pre-incubation, 135 µL of p-nitrophenyl laurate (p-NP-laurate) (2.5 mM, in 5 mM sodium acetate buffer, pH 5.0, with 1% Triton X-100) was added to start the reaction and was incubated at 37°C, for 2 h. The absorbance was measured at 405 nm using a microplate reader. The results were expressed as IC50 values (concentration that inhibits DPP-IV activity by 50%, n = 3).

2.9 Statistical analysis

The results obtained in this study were expressed by the mean ± standard deviation of, at least, triplicates. All data were analyzed by the GraphPad Prism Software (Version 5.0 - San Diego, CA, USA) using analysis of variance (ANOVA), followed by the Tukey test, except where otherwise stated. The value of $p < 0.05$ was considered significant.

3. RESULTS

3.1 Hydrolysis and ultrafiltration

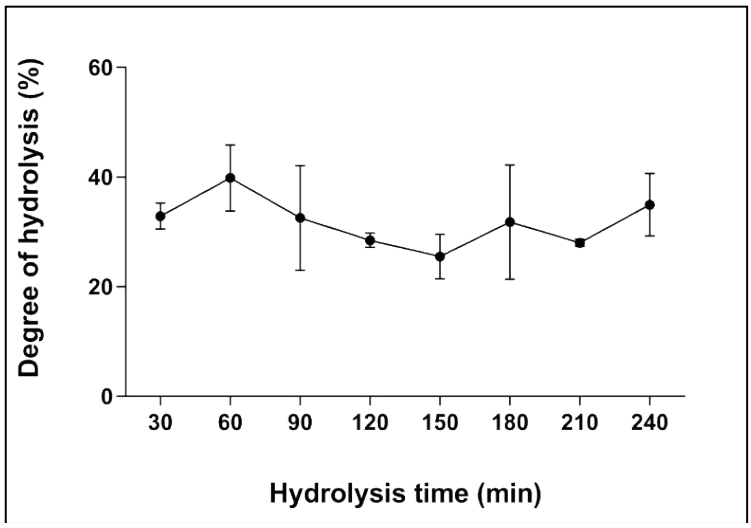

Figure 1. Degree of hydrolysis over time. No significant difference was observed in all times of hydrolysis ($p < 0.05$).

In Figure 1 the degree of hydrolysis over time may be observed. Although the hydrolysis process was conducted in up to 240 min of reaction with aliquots analyzed every 60 minutes, after the initial reaction time of 30 minutes, the maximum yield of 39% DH was achieved after 30 minutes, with no significant difference after this time of reaction. The separation yield was of 16 and 51% for the HMMH and LMMH fractions, respectively, resulting in a loss of 33% of the total peptides after fraction separation.

3.2 Peptides sequencing and *in silico* bioactivity analysis

In the LMMH fraction, 10 larger peptides were identified using the different approaches applied: 3 by matching with the "soybean" database using the Comet software of the PatternLab platform, 1 using the Peaks software and also comparing to the database and 6 using the *de novo* sequencing methodology using the Peaks program. The molecular mass for these peptides ranged from 481.5 to 2,763.21 Da**.** In this same fraction, 15 tripeptides and 7 dipeptides were also identified. These sequences may be observed in the Supplementary Table 2. In the HMMH fraction, 69 peptides were sequenced,

and their molecular masses ranged from 2,996.60 to 6,230.25 Da. Their sequences may also be observed in Supplementary Table 2.

All MS sequences were confronted with the peptide sequences of two of the main proteins found in soy, glycinin and beta-conglycinin, digested *in silico* by the BIOPEP program, in order to identify a possible correlation between them. Fourteen matches were found for the HMMH and 2 for the LMMH larger peptides. These sequences and the cosine value for similarity may also be seen in Supplementary Table 1.

The evaluation of all identified sequences in HMMH and LMMH through BIOPEP-UWM yielded a very rich diversity of possible bioactivities, as can be seen in Figure 2. For the purpose of comparing with *in vitro* analyses, the present work considered the antioxidant activities and those with enzymatic inhibition potential - of α -glucosidase and pancreatic lipase. One hundred thirty-nine fragments with possible antioxidant action were identified in the HMMH fraction and 7 in the LMMH fraction. For α -glucosidase inhibition, the numbers found were lower, 53 and 2 for each respective fraction. No sequence matched inhibition of pancreatic lipase, however, this list in the database is small, it presents only 18 peptides, against 41 sequences of glucosidase inhibitors and 772 antioxidant sequences.

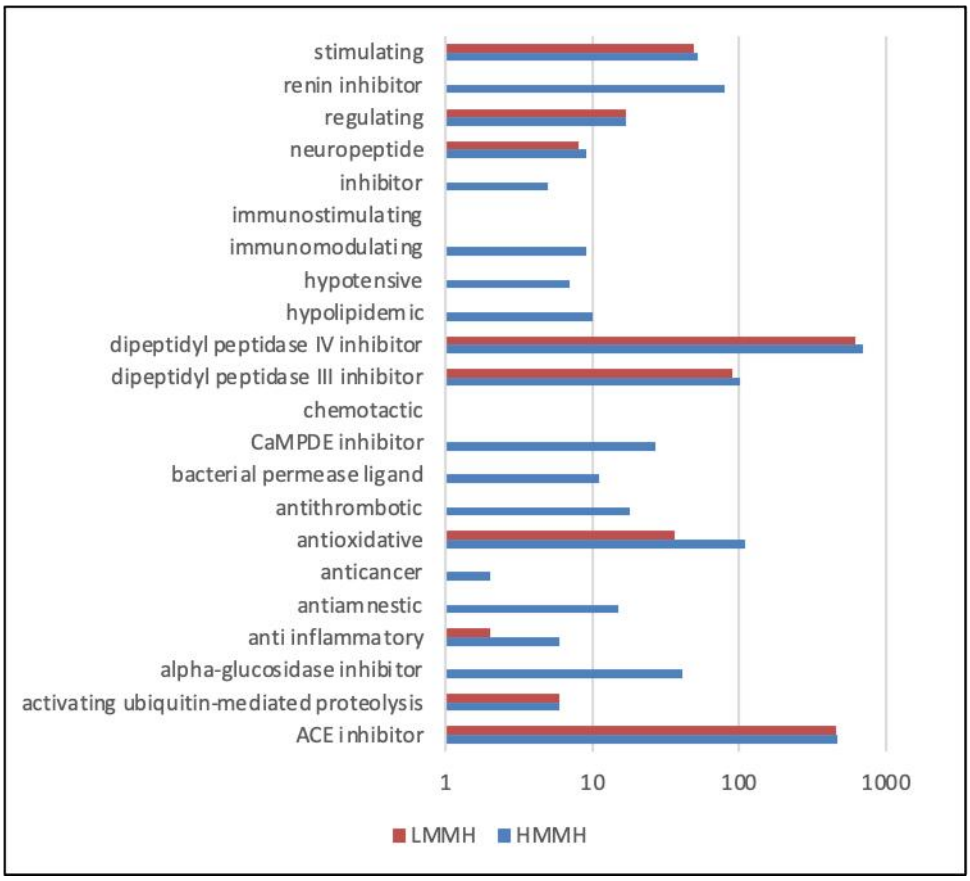

Figure 2. Bioactivities displayed by the identified peptides per number of bioactive sequences located in each sample. (Source: BIOPEP-UWM)

3.2 *In vitro* antioxidant activity analysis

The results related to the antioxidant activity of the hydrolysate fractions are shown in Figure 3, where HMMH showed significantly higher activity, except in the DPPH method.

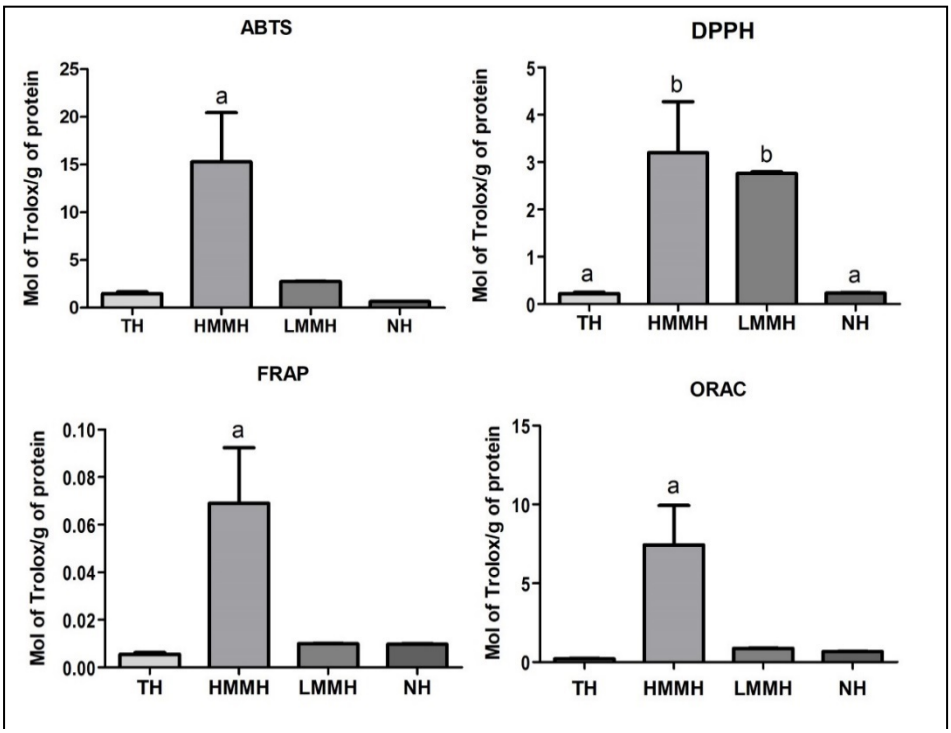

Figure 3. Antioxidant activity of the samples by the antioxidant methods: DPPH, ABTS, FRAP and ORAC. Results expressed as mol Trolox/g of protein. Different letters indicate significant difference ($p < 0.05$). TH – total hydrolysate; HMMH – high molecular mass hydrolysate; LMMH – low molecular mass hydrolysate; NH – not hydrolyzed

In general, the samples behaved in the same way in all analyzes, with one method confirming the other. It is worth remarking that merely hydrolyzing the proteins of the soy protein concentrate applying papain was not enough to significantly increase the antioxidant activity. The separation of the peptides obtained by molecular mass was essential to produce an antioxidant enriched fraction with superior activity compared to the original proteins.

3.2.1 Comparison between *in silico* and *in vitro* analysis - antioxidant activity

To assess whether the prediction of antioxidant activity made using the information obtained by BIOPEP presented a good correlation with the *in vitro* antioxidant activity assays, the normalized A% value was calculated, which was

compared with experimental data of antioxidant activity by the ORAC method. The results may be observed in Figure 4. As can be seen, by the *in silico* method, the fraction with the highest antioxidant activity should be the one with the lowest molecular mass, which was not confirmed by any of the *in vitro* methods applied (Figure 3) and which was significantly different from the data obtained by the ORAC method, used as an example.

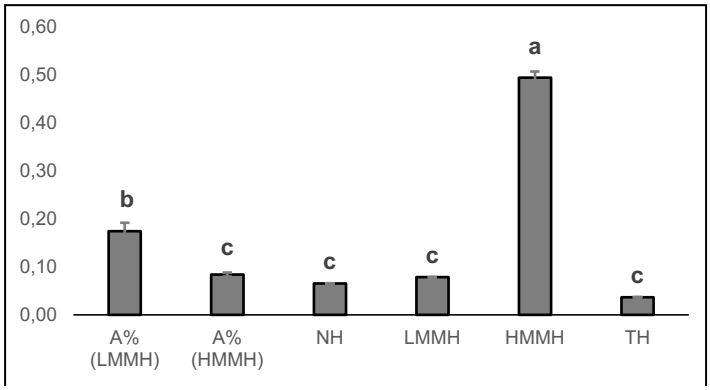

Figure 4. Comparison between biological activity index (A%) and the antioxidant activity evaluated *in vitro* by the ORAC method. Different letters indicate significant difference ($p < 0.05$). TH – total hydrolysate; HMMH – high molecular mass hydrolysate; LMMH – low molecular mass hydrolysate; NH – not hydrolyzed

3.2.2 Application in food model

This assay intended to apply the obtained hydrolysates to a model of antioxidant activity more similar to the application in real foods, compared to *in vitro* methods. For this purpose, homogenized sardine samples were prepared, and the lipid peroxidation of these products was evaluated, over time, in different conservation forms. The TBARS method was used to measure the concentration of the relatively polar secondary reaction products, especially aldehydes, so an increase in the concentrations over time could be observed. It is remarked that the sample treated with HMMH was able to delay the production of these secondary compounds until the last day of the experiment,

when compared to the untreated sample (refrigerated storage), confirming the analyzes of *in vitro* antioxidant capacity previously performed in this study.

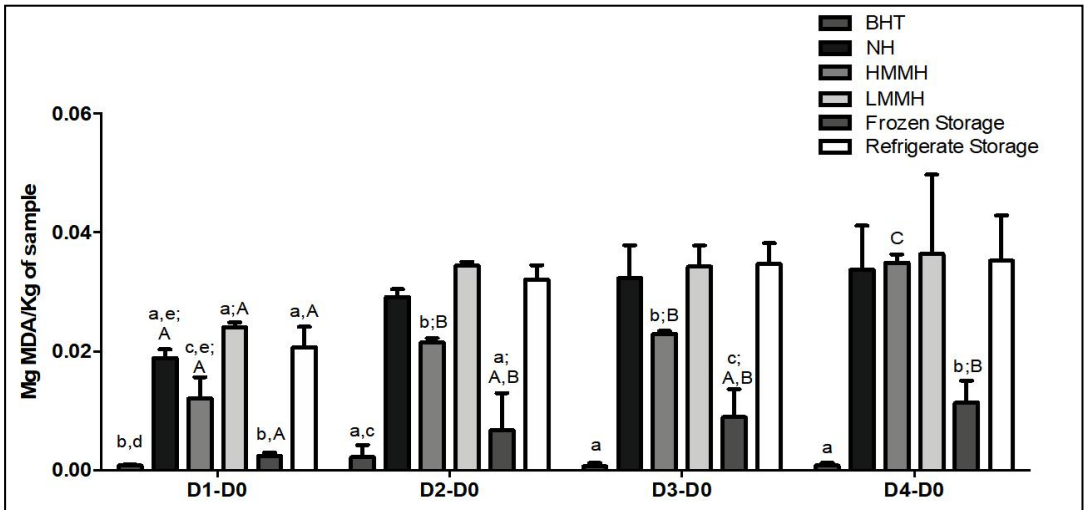

Figure 5. Reaction of TBARS in sardine pates with addition of soy protein hydrolysates. D0 = Analysis after preparation; D1 = 24 hours storage; D2 = 48 hours storage; D3 = 72 hours of storage and D4 = 96 hours of storage. Different capital letters indicate significant difference between the samples over time ($p < 0.05$). Different letters indicate difference between samples at the same time. BHT – butylhydroxytoluene; HMMH – high molecular mass hydrolysate; LMMH – low molecular mass hydrolysate; NH – not hydrolyzed.

As can be observed in Figure 5, the added samples of BHT and the frozen storage obtained the lowest TBARS values, indicating lower lipid oxidation throughout the storage time. This confirms the efficiency of the synthetic antioxidant used and also that freezing is an excellent method for fish products preservation, as already showed by Chaijan et al. (2006) and Veeck, et al. (2013). It may also be observed (Figure 5) that the HMMH was able to delay the peroxidation in the first three times of sampling, with the last point showing no statistical difference in relation to the other samples, except for BHT treated and frozen. These latter samples showed lower values of malonaldehyde, but there was a statistical difference between the third and fourth analysis times, showing the use of BHT to be even more efficient than freezing.

3.3 α-glucosidase and pancreatic lipase inhibition

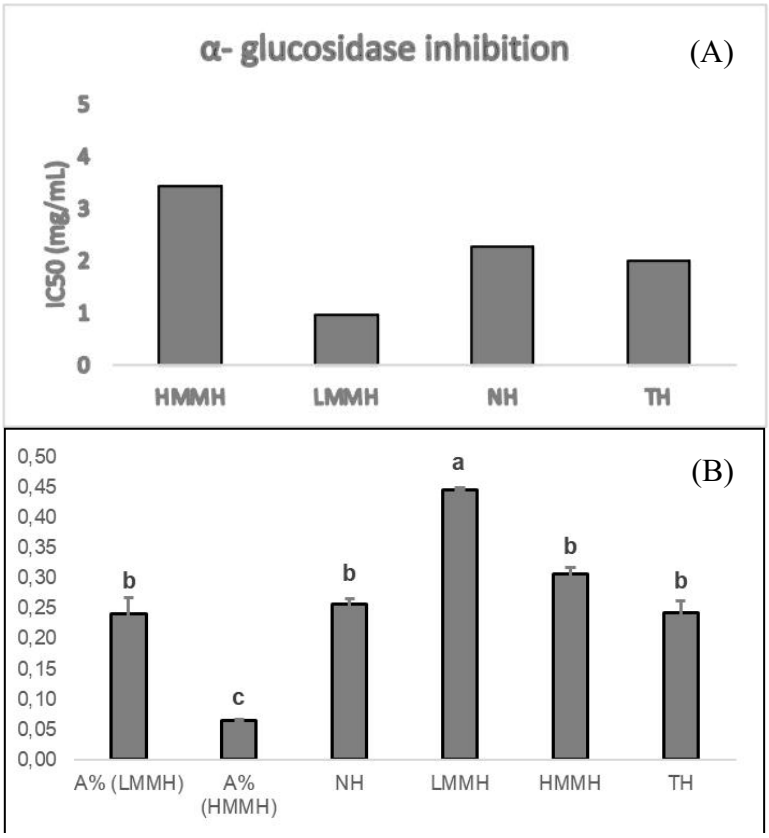

Figure 6. (A) Values are represented as IC50 (mg protein equivalent/mL). No significant difference was observed ($p < 0.05$). (B) Comparison between biological activity index (A%) and the inhibitory activity evaluated *in vitro* considering the concentration of 8 mg/mL. TH – total hydrolysate; HMMH – high molecular mass hydrolysate; LMMH – low molecular mass hydrolysate; NH – not hydrolyzed. Same letters indicate no significant difference between the samples ($p < 0.05$).

The LMMH fraction showed a greater potential for inhibition of α-glucosidase (IC50 = 0,94 ± 5,49) when compared to the HMMH fraction (IC50 = 3,43 ± 1,25), as shown in Figure 6A, indicating that lower molecular mass peptides were more active against α-glucosidase activity. As can also be seen in the same figure (Figure 6B), the normalized biological activity index (A%)

indicated higher inhibitory activity for the LMMH fraction, in agreement with the *in vitro* results.

Regarding lipase inhibition, the best result was obtained by TH sample (IC50 = 1,5 ± 1,77) followed by NH sample (IC50 = 1,9 ± 3,63) (Figure 7). Similar to what happened with α-glucosidase inhibition, the not hydrolyzed sample was also able to inhibit lipase activity.

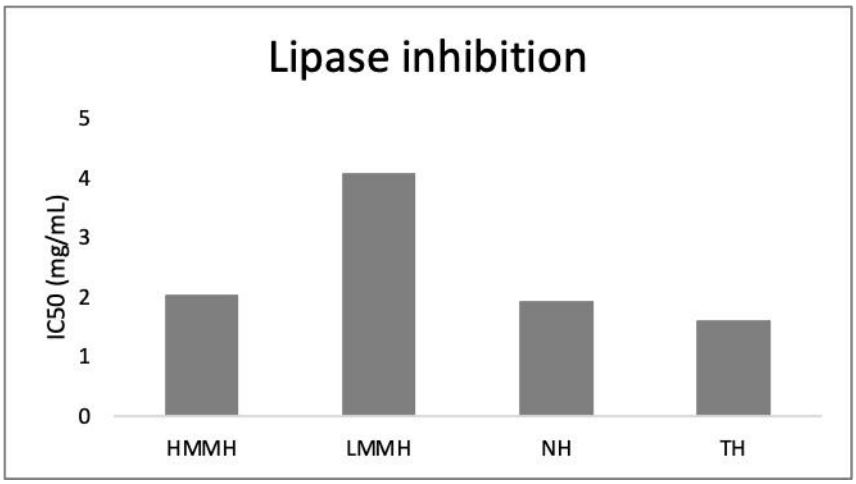

Figure 7. Values are represented as IC50 (mg protein equivalent/mL). TH – total hydrolysate; HMMH – high molecular mass hydrolysate; LMMH – low molecular mass hydrolysate; NH – not hydrolyzed. No significant difference was observed ($p < 0.05$).

4. Discussion

4.1 Hydrolysis and ultrafiltration

Enzymatic hydrolysis is a controlled method that not only improves the biological activity of proteins, but also enhances their functional properties, such as digestibility (Barati et al., 2020). In the present study, similarly to what was reported by Cotabarren et al. (2019) and Borrajo et al., (2020), when the reaction time was extended, no significant increase in DH was observed. Peredo-Lovillo et al., (2022) in a recent review, observed that papain reaction times ranged from 40 to up to 480 minutes of hydrolysis with comparably good

results. These studies suggest that, in order to obtain peptides with a specific bioactivity, the selection of the enzyme (specificity) and the nature of the protein source should be considered first rather than the reaction time or the degree of hydrolysis (Peredo-Lovillo et al., (2022). Enzymatic hydrolysis of defatted green soybean meal using Alcalase and papain performed by Hafani et al (2018) showed a rapid increase in the degree of hydrolysis during the first 30 minutes, corroborating the data of this work. The authors also observed that the reaction rate decreased over time, which can be explained by the inhibition of the enzyme by the cleaved protein products; depletion of available hydrolysis sites or by inactivation of the enzyme under the reaction conditions (Hafani et al., 2018).

Regarding the molecular mass distribution after hydrolysis, Tong et al. (2020) reported 64.9% of peptides with up to 5 kDa in a study about soy hydrolysates obtained with Protex 6L, after a 2-hour reaction. Zhang et al (2021), in turn, identified 16.21% in molecular mass between 1-3 kDa and approximately 78% less than 1 kDa showing with soy hydrolysates with Alcalase after ultra-sound treatment. No mass distribution information was found about soy hydrolysates obtained with papain, but a certain pattern may be pointed out, in which even fast reactions with different proteases tend to generate a greater fraction of low molecular mass peptides.

4.2 Differences between *in silico* x *in vitro* analysis – soy protein hydrolysis

In the present work, 101 peptides were found after hydrolysis of SPC with papain and analysis by LC-MS/MS, while *in silico* hydrolysis of the main (most abundant) storage proteins using the theoretical specificity of papain

generated 118 unique peptides. Of these totals, only 16 were matches with a reasonable degree of similarity. Theoretical hydrolysis using bioinformatics tools, including BIOPEP-UWM, is based on enzyme specificity and substrate protein sequence. Hypothetically, in this process, all peptide bonds are accessible to enzymatic cleavage, which does not consider the existence of overlapping sequences within specific regions of protein molecules, the inaccessibility of a peptide bond of the substrate for enzyme attack, the substrate interaction with another protein, the impact of inhibitors, among other factors (Iwaniak et al., 2019; FitzGerald, et al. 2020; Iwaniak et al., 2020). This can be illustrated by the findings of Gibbs et al. (2004) who showed that 95% of soy peptides generated by *in vitro* hydrolysis came from their globulin glycinin (11S), and that β-conglycinins (7S) were more resistant to hydrolysis, even applying multienzymatic cocktails, which is most probably not taken into account by the algorithms that perform the theoretical hydrolysis. Furthermore, such predictive hydrolysis does not consider chemical modifications of the proteins. For example, glycosylated amino acids present in a protein can block the lysis of a peptide bond under experimental conditions. According to the bibliometric review by Barati et al. (2020), when matching with spectra from databases is the method used to identify sequences analyzed by MS, the result is successful if the target protein database is complete, and cleavage performed by digestive enzymes is absolutely clear. Otherwise, the probability of identifying related peptides is considerably reduced. Therefore, the continuous need to feed the database is so important, both for bioactive peptide sequences and for the enzymes and proteins used in the process of obtaining them (Iwaniak et al., 2019; FitzGerald, et al. 2020; Iwaniak et al., 2020).

The availability of data recorded in databases may also be responsible for the high number of bioactive sequences matches. In the present work, for antioxidant activity, 146 match sequences were found (Figure 2), while in the study by Silva do Nascimento et al. (2021) only 33 matches were registered in the same database (BIOPEP-UWM). This difference may have various causes: the source of protein under study (yam) and/or the type of hydrolysis evaluated (simulated digestion) may result in a lower number of antioxidant peptides than the papain hydrolyzed soy proteins. However, there is a reasonable probability that a lack of information in the database about yam proteins and peptides is responsible for the lower number of bioactive sequences. On the other hand, the work by Cui et al. (2022), investigated the production of bioactive peptides from milk proteins, via fermentation. Cow's milk proteins are among the most studied for obtaining bioactive peptides and Cui et al. (2022) were able to match only between 33 and 55 (varying with the fermenting microorganism) antioxidant sequences by searching the same database.

Regardless of possible analytical difficulties, the bioactivities found for soy peptides, especially those of low molecular mass (LMMH), in the present study, were also found by Crozatti et al. (2022) and by Tamam et al. (2020), even if using different enzymes (Alcalase, Neutrase), namely: ACE inhibitor; dipeptidyl peptidase iv inhibitor; stimulating; renin inhibitor; CaMPDE inhibitor; antioxidant, which indicates the existence of sequences with these bioactivities encrypted in soy storage proteins and the ability of different enzymes to release them by hydrolysis.

4.3 Differences between *in silico* x *in vitro* analysis – antioxidant activity

The enzymatic hydrolysis of proteins is the main process to generate bioactive peptides with antioxidant capacity (Sbroggio et al., 2016). The antioxidant activity of proteins is recurrently related to their amino acid composition; however, this property of the residues is limited by their interactions in the tertiary structure. Many potential antioxidant residues are located within the protein core, where they are inaccessible to pro-oxidants. Therefore, the hydrolysis tends to increase the activity by exposing the antioxidant amino acids in the proteins (Carocho et. al., 2018).

The *in silico* analysis for the antioxidant activity predicted the occurrence of higher activity for the LMMH than for the HMMH, in agreement with different articles in the literature that credit higher antioxidant activity to low molecular mass peptides (Ji et al., 2014; Jang et al., 2016). Some recent works on *in silico* methodologies report that *in vitro/vivo* analyzes often do not correspond to those found in databases and may suggest 'optimistic' results that differ when verified under laboratory conditions, and vice-versa. In Iwaniak et al. (2020), for example, the percentage of compatibility of the *in silico* and *in vitro* results for the bitter taste of soy peptides was less than 3%

Although low molecular mass peptides often relate to high antioxidant activity, there is a lack of evidence to indicate the exact range of molecular mass that encompasses the most potent peptides (Tang, 2014; Samaranayaka and Li-Chan, 2011). According to Tang et al. (2010), when studying the antioxidant activity of zein hydrolysates, low molecular mass peptides showed higher affinity for the DPPH radical. That may explain the high antioxidant capacity detected by this method in the LMMH fraction (Figure 3). However, in a

study by Zhou et al. (2012), in which the antioxidant activity in hydrolysates obtained from shellfish viscera by different proteases was evaluated by the DPPH method, the most effective peptides were in a wide range of molecular mass, and the lower mass peptides did not present higher antioxidant activity, as initially expected by the authors. Xia et al. (2012) also showed that barley glutelin peptides with molecular mass > 10 kDa presented higher antioxidant activity than the lower mass fractions when tested by the DDPH method. The authors explained this behavior by the formation of glutelin-specific hydrophobic agglomerates, with voluminous and aromatic side chains, which could be acting as hydrogen donors and as direct radical scavengers.

The other three methods used in the present study showed behavior consistent with each other (Figure 3). Essentially there is not much difference between the ABTS assay and the FRAP assay because they both use the same mechanism of action (electron transfer), the only difference is that ABTS is performed at neutral pH while FRAP under acidic conditions (Comert and Gokmen, 2018). On the other hand, Phetatisit et al. (2018) conducted a study comparing the ORAC and the FRAP assays on concentrates of different proteins, including soy protein, and found no correlation between the two methods, concluding that ORAC was the best indicator of the two for the antioxidant activity. Because of these differences, more than one assay should be and often is used to measure the antioxidant activity of a food or food constituent of interest (Tang, 2014). The observations in the present study indicated that the molecular mass may not be the most important factor influencing the antioxidant activity of hydrolysates or peptides. Nevertheless, the importance of small peptides should not be overlooked and may be

estimated by observing the BIOPEP database, where there are 496 dipeptides and 573 tripeptides registered as bioactive. Studies with synthetic di- and tripeptides suggested that they are able to avoid the intestinal barrier, crossing from the lumen into the blood system, with preserved activity, whether antihypertensive, antioxidant, among others (Sanchez et al., 2017; Poliseli et al., 2021).

The amino acid composition, specific peptide properties, reaction media and pH may play a more crucial role in the antioxidant activity of peptides than their molecular mass (Sanjukta and Rai, 2016; Samaranayaka and Li-Chan, 2011). As early as 1996, Chen et al. verified that the presence of histidine (H) and proline (P) were crucial for the antioxidant activity of the peptide LLPHH obtained from soy hydrolysates. More recently, Dai et al. (2017) and Coscueta et al. (2019) were able to relate strong antioxidant activity to the peptides TTYY and LLPHHADADY, respectively, also found in soybean hydrolysates. In the present study, both these sequences were identified in different peptides of the HMMH fraction. This may be related to the strong antioxidant activity displayed by this fraction (Figures 3 and 4), as peptides containing histidine and aromatic amino acids such as tyrosine (Y), tryptophan (W) and phenylalanine (F) are able to act as metal chelators as well as radical hydroxyl and/or reactive oxygen species scavengers (Mejia; Lumen, 2006; Pihlanto, 2008; Lie et al., 2016; Sompinit et al., 2020). On the other hand, according to Meija and Lumen (2006); Elias et al. (2008), and Sbroggio et al. (2016), the most antioxidant amino acids, beyond the ones presenting aromatic side chains (W, Y and F), are those with nucleophilic sulfur-containing side chains (C and M). Among the sequences marked as antioxidant by the BIOPEP search in the HMMH fraction,

35 presented V and/or I and 90 presented F, Y, W and/or H as part of their antioxidant selected sequences, indicating that these large and aromatic residues may be acting as antioxidant. Although many of the peptides identified presented C and/or M within their sequences, none of these portions were marked as antioxidant by the search on BIOPEP-UWM.

4.3.1 Application in food model

Lipid oxidation is induced by the generation of free radicals and results in the generation of toxic compounds such as malondialdehyde and cholesterol oxidation products. However, proteins (peptides and amino acids) in soy protein concentrate have multi-antioxidant activity (Dellafiora et al., 2020). Thus, these compounds could scavenge free radicals and reactive oxygen species, resulting in the prevention of lipid oxidation and some studies have already demonstrated the potential to delay lipid peroxidation linked to antioxidant peptides (Wang et al., 2017; Li et al., 2016; Nasri et al., 2013). In a study by Penã-Ramos and Xiong (2003), analyzing soy and milk hydrolysates obtained by enzymatic hydrolysis, it was observed that soy hydrolysates were able to inhibit the peroxidation of cooked pork pates over seven days more effectively than milk hydrolysates. As these results were somewhat unexpected, the authors attributed this effect to the antioxidant phenolic compounds usually present in soy protein isolates. Another study on soy peptides, obtained by enzymatic hydrolysis by microbial proteases, this time with ground beef, was conducted by Zhang et al. (2010). The hydrolysates with molecular mass over 10 kDa showed antioxidant activity, delaying peroxidation for 15 days of experiment, when compared with the control. Oliveira et al. (2014) administered isolated soy

protein, after a 6-h hydrolysis by bacterial protease, in two different meat products containing pork or salmon, at concentrations of 2 and 10 mg/mL. Peroxidation inhibition was of 11.74% and 65.09% for salmon and 46.70% and 62.64% for pork, compared to the control, without the addition of hydrolysates. In the present study, the samples in the model-food test behaved as it was estimated by the *in vitro* antioxidant activity evaluation methods and inversely to what was predicted by the *in silico* evaluation: HMMH > LMMH (Figure 4), indicating that *in vitro* tests may be well correlated with the application of antioxidant peptides in foods. In this test, HMMH was able to significantly delay the peroxidation of sardine pates for 3 days of storage, compared to a control with no added antioxidant, showing to be a promising natural additive for animal products with a high risk of lipid oxidation

4.4 Peptides sequencing and *in silico* x *in vitro* bioactivity analysis – digestive enzymes inhibitory activity

Different from what happened with the results of antioxidant activity, the use of the normalized biological activity index (A%) for predicting α-glucosidase inhibitory activity generated results similar to that obtained with the *in vitro* analyses. This may indicate that the factors responsible for the inhibition of α-glucosidase by peptides are more constant than those responsible for the antioxidant activity, facilitating matches. Recent studies have worked with the comparison of *in vitro* methods and *in silico* analysis in the so-called integrated analysis (hybrid) with results that complement each other (Senthil et al., 2019; Ibrahim et al., 2018). However, the *in silico* analyzes are considered preliminary approaches, expanding the knowledge and allowing the planning of the experiment (Peredo-Lovillo et al., 2022; Iwanniak et al. 2019).

Enzymes associated with the regulation of postprandial hyperglycemia, such as α-glucosidase, α-amylase, and dipeptidyl peptidase IV (DPP-IV), have been recognized as therapeutic targets. The guideline of the American Diabetes Association and the European Association for the Study of Diabetes has recommended the use of α-amylase and α-glucosidase inhibitors as potential first-line agents and in combination with other anti-hyperglycemic drugs. Bioactive peptides, particularly from natural products, already showed high potential for application in the management and treatment of diabetes (Yan et al., 2019; Wang et al., 2019). The beneficial effects of these inhibitory peptides can be explained through several mechanisms, such as the satiety response, regulation of incretin hormones, insulinemia levels, and reducing the activity of carbohydrate degrading digestive enzymes, which is positively related to suppress hyperglycemia in diabetes *mellitus* (Han et al., 2021). In the present work, LMMH and TH showed the best values of IC50 (0,94 and 1,99 respectively) on α-glucosidase inhibition, although it was also observed that the not hydrolyzed and the HMMH peptides samples also exhibited activity, reinforcing that soybean show great potential as an α-glucosidase inhibitor, also indicated elsewhere (Lam et al., 2020; Chen et al., 2021). Wang et al (2019) showed a significant increase in the α-glucosidase inhibition function after intestinal digestion in soybean hydrolysates. These results demonstrated that the peptides could endure the whole digestive environment and still maintain or enhance their bioactivity. This was also shown for antioxidant activity by Coscueta et al (2019). Gonzalez-Montoya (2018), testing soy peptide fractions of size between 5-10 kDa, identified inhibitions between 27 to 36%, suggesting that peptides of different molecular masses may show synergistic or additive

inhibitory effects, which might explain the high inhibitory activity of the TH sample. According to the authors, inhibitory peptides mainly bind to the catalytic domain of α-glucosidase through hydrogen bonds and electrostatic interactions. In addition, other structural features such as a net peptide charge of 0 or +1, the presence of basic and sulfur-containing amino acid residues and hydrogen bonds significantly increased the potency of α-glucosidase inhibitor peptides, while negatively charged peptides showed reduced inhibitory potency. Jiang et al. (2018) identified two tripeptides with high potential for inhibiting α-glucosidase in soy hydrolysates and their molecular docking analysis revealed that the α-glucosidase-inhibitory activity of GSR and EAK can be mainly attributed to the four and five hydrogen-bonding interactions, respectively, between these two tripeptides and α-glucosidase. None of these sequences appeared in our data, but the sequence EA was showed 9 times as responsible for α-glucosidase inhibition in the HMMH fraction as indicated in Supplementary Data 2. Barati et al. (2020) also reported that AKSPLF, QTPF, FEELN and LSKSVL were known as multifunctional antidiabetic peptides due to their potential inhibitory activities of DPP-IV, α-amylase, and α-glucosidase.

The inhibition of pancreatic lipase by peptides is much less studied than the inhibition of α-glucosidase and the mechanisms of this kind of lipase inhibition are still poorly understood, as may be verified by the lesser number of published articles on the subject and the little information about soy-based lipase inhibiting peptides available in the BIPOEP-UWM database.

The mechanism of lipase inhibition is based on impairing the catalytic role of lipase in triglyceride hydrolysis and inhibition of fat breakdown into monoglyceride and fatty acids (Younis et al., 2021). Ajayi et al. (2021) reported

that among 17 bioactive peptides identified in amaranth, three (FPFPTLGY, FGAPR, and FPFVPAPT) were predicted to be potential pancreatic lipase inhibitors. The mechanisms whereby soy protein may exert its beneficial effects on obesity are not completely clear. Several lines of evidence suggest that soy protein may favorably affect lipid absorption, insulin resistance, fatty acid metabolism, and other hormonal, cellular or molecular changes associated with adiposity (Velasquez, 2007). Synthetic soybean peptides (EITPEKNPQLR and RKQEEDEDEEQQRE) showed a manner to block the catalytic domain of the enzyme fatty acid synthase (FAS) inhibitory activity, which plays a key role in the endogenous synthesis of saturated long-chain acetyl-CoA and malonyl-CoA precursor fatty acids (Martinez-Villaluenga et al., 2010). There are still very few results reported on pancreatic lipase inhibition by soy peptides. The recent study by Alnuaimi et al. (2023) reported 11 peptides from young and mature soy proteins with pancreatic lipase and cholesterol esterase inhibition activity, of which two were matched to sequences found in the HMMH sample – ***FPLL***VLLGTVFLASVCVSLKVREDENNPFYFR and FFEITPEKNPQLRDLDIFLSSVDINEGALL***LPHF***NSK. This indicates that these peptides may be the ones responsible for the lipase inhibition activity in this sample, which showed lower IC50 values when compared to the LMMH sample. However, further studies are needed to better understand the molecular mechanisms of action of these peptides and to feed the *in silico* databases.

5. CONCLUSION

In the present work, a protein hydrolysate from soybean protein concentrate, an abundant plant protein, was produced using papain, a readily

available commercial protease, which has been little applied in obtaining this type of product, in a simple, time- and cost-effective manner. Molecular mass fractionation of the hydrolysate proved to be indispensable for the detection of antioxidant activity by the in *vitro* methods, but not for the inhibitory action on α-glucosidase and pancreatic lipase. The high molecular mass fraction (HMMH) was able to delay the lipid oxidation of sardine homogenate under refrigeration, in addition to inhibiting the action of α-glucosidase (IC50 = 3,4) and of pancreatic lipase (IC50 = 2,02). This multiple activity would allow the application of this ingredient in the conservation of meat products with the possible additional effect of delaying the glycemic peak caused by the hydrolysis of carbohydrates ingested at the same meal (hot dog; meatball sandwich; pasta with meatballs or sausages, for instance) and reducing the digestion/absorption of lipids consumed in the diet as well. The lower molecular mass fraction (LMMH), although not indicated as an antioxidant, showed potential for inhibiting α-glucosidases (IC50= 0,94) and lipases (IC50 = 4,06), presenting the possibility to act as an ingredient to delay the digestion of carbohydrates, helping to control glycemia and/or prolonging the energy supply in possible formulations indicated for endurance athletes.

The use of different analytical methods of shotgun peptidomic allowed the elucidation of the structure of 109 different peptides present in the hydrolysate, ranging from 481.5 Da to dipeptides, which were related to more than 20 different types of bioactivities through the BIOPEP-UWM database search. *In silico* bioactivity evaluation correlated poorly with antioxidant *in vitro* testing, but well with *in vitro* α-glucosidase inhibition. This was, to the best of

our knowledge, the first report of soy hydrolysate related pancreatic lipase inhibitors.

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7. CONFLICT OF INTEREST:

We declare no conflict of interest.

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9. AUTHORS CONTRIBUTIONS

FARIAS, T. C. – Investigation, Software, Formal analysis, Writing, Visualization; ABREU, J. P. – Investigation; OLIVEIRA, J. P. S. – Software, Formal analysis, Visualization; MACEDO, A.F. – Resources; RODRÍGUEZ-VEGA A. – Investigation; TONIN, A. P. – Investigation; CARDOSO, F. S. N. - Formal analysis, Visualization; MEURER, E. C. – Methodology, Supervision, Visualization; KOBLITZ, M. G. B. – Conceptualization, Resources, Supervision, Writing, Visualization.

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CAPÍTULO IV

ANTIPROLIFERATIVE EFFECT OF PEPTIDE FRACTIONS FROM BRAZILIAN SOY PROTEIN HYDROLYSATES IN PROSTATE CANCER CELLS

A ser submetido para: "Nutrition and Cancer"
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ABSTRACT

Bioactive peptides are oligopeptides that mostly consist of 2–20 amino acids that can exert beneficial effects on human health in addition to basic nutritional effects. Food derived protein hydrolysates or peptides with anticancer activities were expressed in literature. The aim of this study was to evaluate the influence of soybean bioactive peptides on cell proliferation, cell cycle, and apoptosis rate of human prostate cancer cell lines and identify the possible sequences responsible for this activity by searching *in silico* databanks. Initially soy protein concentrated was hydrolyzed with papain and fractionated by ultrafiltration which produced two fractions (LMMH and HMMH) that were compared with the non-fractionated (TH) and non-hydrolyzed (NH) samples. The sequences obtained by mass spectrometry were confronted on BIOPEP-UWM database and two tri-peptides in the HMMH fraction showed the Val-Val-Val sequence, known for presenting anticancer activity. All samples showed antiproliferative activity up to 50% against PC-3 cells at 48 h. TH and NH induced cell cycle arrest in the G0/G1 phase. Further, TH and LMMH induced the apoptosis of PC-3 cells. Thus, soybean hydrolysates may be a good source for providing antiproliferative peptides against PC-3, while the TH using papain may have the potential to be developed as a nutritional supplement in an alternative cancer therapy. Therefore, further studies should be carried out to identify the structure of the peptides responsible for this activity and also to feed the peptide bioactivity database to favor future studies.

Keywords Bioactive peptides; Cytotoxicity; MTT assay; Cancer cell lines; Soybean

1 Introduction

In Brazil, prostate cancer is the second most common among men (after non-melanoma skin cancer). In absolute values and considering both sexes, it is the second most common type. The incidence rate is higher in developed countries compared to developing countries. According to an estimate by the National Cancer Institute (2020) (1), prostate cancer has reached more than 65,000 cases in men in Brazil, with an incidence of approximately 30% of cancer cases. Statistically, one in six men will develop some form of prostate cancer in their lifetime, and interestingly, almost 50% of men have tumors within their prostate upon autopsy (2).

Soybean (*Glycine max*) is an important legume that has been extensively studied due to its many biological functions in the body, including anticancer activity. The most studied bioactive substances are isoflavones, the Bowman-Birk protease inhibitor (BBI) and the less purified BBI concentrate (BBIC), in addition to proteins and peptides (3). In recent years, many studies have focused on soy-derived bioactive peptides, which are natural peptides or small protein fragments produced by hydrolysis, fermentation or maturation, among which enzymatic treatment is the most suitable for the preservation of functional and nutritional values of the protein hydrolysate (4). Most epidemiological studies have suggested that the consumption of soy food is associated with a reduction in prostate cancer risk in humans. These findings suggest that this protection is related to the type and quantity of soy foods consumed (5;6).

Soy proteins contain all essential amino acids and therefore represent a valid alternative to foods of animal origin. Moreover, their peptides have been identified with multiple biological functions, including hypolipidemic, antidiabetic, anticancer and antihypertensive effects (7-11). Recently, proteins and peptides from various plant or animal proteins, especially milk, soybean or fish proteins, have been shown to have antitumor or antiproliferative activities (12).

The chemopreventive effects of soy have been attributed to its bioactive molecules, such as proteins, peptides, saponins, and isoflavones, which can interact with metabolic pathways and signaling cascades that control cell growth, proliferation and differentiation, cell survival or death, and inflammation induced by lipopolysaccharide (LPS) (13). Soy naturally contains a peptide named Lunasin, which has been extensively studied and has a known anticancer function (14-15). Anticancer peptides performs their function by the amino acid sequence, net charge, amphipathicity,

hydrophobicity, structural folding (including secondary structure, dynamics and orientation) in membranes, oligomerization, peptide concentration, and membrane composition. They are normally characterized by low weight, usually less than 30 amino acids, and exhibit a predominantly cationic amphipathic structure, making them disposed of molecular membrane surfaces (16).

Controlled enzymatic hydrolysis can generate peptides that can elicit bioactivity including anti-proliferative action against cancer cell lines. Studies have indicated that soybeans are excellent sources of pure bioactive peptides, including anticancer activity with more than 80% inhibition of human colon and blood cancer cells and 68% in blood cancer cell attributed to peptides between 10 to 50 kDa (17-18). Another study with soybean hydrolysate with bromelin and thermolysin showed 35.45% to 76.39% of inhibition in HSC-3 human oral with no cytotoxic effect in normal oral cells lines, respectively (12). Other soybean hydrolyzed products were tested against various types of cell lines like CaCo2 (19), MDA-MB-231 human breast cancer (15), human liver (HepG2), lung (MCF-7) and cervical (Hela) (20), showing promising effects.

The lower incidence of prostate cancer in some Asian countries has been explained by the high consumption of soy foods in these populations. The protective effect of soy foods against the development of prostate cancer could be the result of both, hormonal and non-hormonal, effects of soy foods (5). A recent literature review about prostate cancer and soybean intake indicated that the protective effect of soy foods could be related to other mechanisms as antioxidant effect or reduction of tumor cell growth by induction of apoptosis or inhibition of angiogenesis, as the authors indicated that the protective mechanism is not fully understood in *in vivo* studies (21).

Utilization of the relatively low-cost soybean protein to produce effective peptides against proliferation of cancer cells is the significant achievement of this research. In the present study, for the first time in literature, the influence of soy protein hydrolysates obtained with papain enzyme, at different ranges of molecular weight, was evaluated on cell proliferation, cell cycle and apoptosis rate of human prostate cancer cell lines (PC-3).

1. Methodology

1.1 Plant, enzyme and food material

Soy protein concentrate (SPC) containing 75% of total protein was obtained in the local market. The purified proteolytic enzyme (papain EC 3.4.22.2) used was purchased from Sigma®. The enzyme:protein (E:P) ratio was set at 1:80, according to previous tests (data not shown).

2.2. Hydrolysis

Hydrolysis was conducted based on the modified method by Contreras et al. (22). Briefly: samples of 2.7 g of SPC were added to 100 mL of phosphate buffer (50 mM; pH 7.0) and heated at 95 °C, for 15 min then cooled and kept in a bath at 60 °C. The addition of protease initiated the reaction. After 30 minutes, the medium was heated at 100°C/10 min., cooled and centrifuged at 13,000 x g for 15 min. (Thermo Fisher Scientific, USA). The supernatant was aliquoted and freeze dried. A not hydrolyzed sample (NH), where no enzyme was added, was used as control.

2.3. Ultrafiltration

After hydrolysis, the total hydrolysate (TH) was separated by ultrafiltration process applying centrifuge devices (Amicon Ultra-4 Filters with Ultracel-3 membrane, Millipore, USA). Thus, fractions of the hydrolysate containing peptides of molecular masses (MM) of < 3 kDa (LMMH - low molecular mass hydrolysate) and > 3 kDa (HMMH - high molecular mass hydrolysate) were obtained. The samples produced were freeze dried and stored at -18ºC until use.

2.4 Peptides identification

The peptides were identified by protein digestion and analysis by liquid chromatography-mass spectrometry (LC-MS/MS). MS acquired sequences that were successfully remapped to potential storage proteins were processed by the online BIOPEP-UWM tool (Minkiewicz et al. (23), https://biochemia.uwm.edu.pl/en/biopep-uwm-2/) to assess if the *in vitro* tested activities were related to them through an *in silico* approach, following the methodology described by our previous work (24).

2.5. Cell assays

Prostate adenocarcinoma cell lines PC-3 were obtained from Cell Bank of Rio de Janeiro (Parque Tecnológico de Xerém, Duque de Caxias). PC-3 is a prostate cancer bone metastasis cell line (25). The prostate cells were cultured in RPMI medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% amphotericin, pH 7.4, under 5% CO_2 atmosphere and 37 °C temperature. Controls used in cell assays were the cell lines in the same medium without any peptide or protein sample (26).

2.5.1. MTT cell viability assay

Cell viability was determined using the MTT (3- [4,5-dimethyl- thiazol-2-yl] -2,5-diphenyl-tetrazolium bromide) assay (27). The cells were plated in 96-well microplates with 2.0×10^4 cells/well. Cells were treated with eight distinct concentrations of the samples (0,3; 0,625; 1,25; 2,5; 5; 10; 20 and 40 mg/mL) and then incubated for 48 hours. Ten microliters of MTT solution (5 g/L) were added to each well. After incubation for 2 h, the culture medium was removed and 50 μL of dimethyl sulfoxide (DMSO) were added to solubilize the formazan. Samples were read in an ELISA reader (Bio-Rad iMARK) at 570 nm. Cell viability was calculated comparison with the control (100 %).

2.5.2. Cell cycle analysis

Cells were plated in 6-well microplates with 5.0×10^5 /well. Prostate cancer cells incubated for 48 h in the presence and in absence of the four samples ((at concentrations 10 mg/mL and 20 mg/mL)) were detached using trypsin solution at 25 °C. The cell suspension was analyzed for DNA content by a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Vindlov's reagent was used, to read 30,000 cells for each replicate (28). Cells with lower DNA content than G1 in the cell cycle distribution were considered hypodiploid cells (subG1). Relative proportions of diploid G0/G1 (2n), S (>2n, but < 4n), and G2/M (4n) indicative of DNA content were acquired using Cell Quest iPro. The percentage of cell population in each specific phase was estimated with the FlowJo v 10.0.6 software and compared to the control.

2.5.3. Apoptosis assays

Phosphatidylserine externalization was observed through the Annexin-V assay using the flow cytometry technique (29) to indicate the percentage of cells that were probably viable, in apoptosis or non- apoptotic cell death, following the methodology of Montenegro et al (30). Prostate adenocarcinoma cells were incubated in a 6-well microplate using 5.0×10^5 cells/well with the extracts for 48 h. The cells were detached using a trypsin solution, and then the propidium iodide and annexin markers were added. Detection was carried out with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) using the Cell Quest iPro software, to count 30,000 cells units/ replicate. The cell populations analyzed were recognized by their forward scatter (FSC)/side scatter (SSC) properties. Fluorescein isothiocyanate (FITC) green fluorescence was measured at 530 ± 30 nm (FL1 detector) and propidium iodide red fluorescence was measured at 585 ± 42 nm (FL2). The percentage of viable cells, and those cells in early or late apoptosis or non-apoptotic death was calculated using the FlowJo v 10.0.6 software.

2.6. Statistical analysis

All assays were performed in triplicate and results were expressed by mean ± standard deviation. Data were analyzed using GraphPad Prism (version 5.04, GraphPad Software, San Diego, CA, USA). Results were compared by one-way analysis of variance (ANOVA) together with the Tukey post-test with a confidence level of 95%. In cell cycle and apoptosis assays 99% and 99.9% of confidence were tested as well.

2. Results and discussion

3.1 Peptides identification

By the identification of the fractions 10 larger peptides, 15 tripeptides and 7 dipeptides were identified in the LMMH fraction with molecular masses between 481.5 to 2,763.21 Da. In the HMMH fraction, 69 peptides were sequenced, and their molecular masses ranged from 2,996.60 to 6,230.25 Da. The evaluation of all identified

sequences in HMMH and LMMH through BIOPEP-UWM yielded a very rich diversity of possible bioactivities. But only two sequences were matched to anticancer activity, both located in the HMMH fraction and both showing three valine sequential residues (VVV). All the sequences that were matched to bioactivities may be observed in Supplementary Table 1. According to some studies, the hydrophobic amino acids like Phe (F), Ile (I), and Val (V), may enhance interactions between anticancer peptides and the outer leaflets of tumor cell membrane bilayers, and thereby exert selective and stronger cytotoxic activity against cancer cells. It has been shown that the presence of charged (glutamic acid) and heterocyclic amino acid (proline) in the sequence could also contribute to anti-cancer properties of the peptides (19; 31). Gonzalez-Montoya et al. (13) identified that peptides from germinated soybean with the presence or rich in glutamine (Q) and serine (S) could be responsible for anticancer and anti-inflammatory activities. Rayaprolu et al (17) identified that the peptide fraction of hydrolysis of soybean resulted in a potent single peptide of molecular size of 18 kDa with 158 amino acid residues and with highest anti-proliferative activity against the colon cancer cell line tested.

Peptides may act against cancer cells through different mechanisms including: stimulation of apoptosis, cell cycle progression arrest, cell membrane damage, inhibition of cell adhesion, topoisomerases, modulation of immune response, and inhibition of intracellular signaling. Table 1 shows soybeans peptides and hydrolysates showing activity against different cells cancer lines.

Table 1 – Types of cancer in different soy products and peptides in recent literature.

Source	Cell line	Reference
Lunasin	MDA-MB-231 human breast cancer	Hao et al. (15)
Lunasin	HT-29 colon cancer	Dia et al. (32)
Black soybean protein hydrolysate (Alcalase)	Human liver (HepG2), lung (MCF-7) and cervical (Hela) cancer	Chen et al. (20)
Soybean seeds (Alcalase)	Blood (CCRF-CEM and Kasumi-3), breast (MCF-7), and prostate (PC-3) cancer cell	Rayaprolu et al. (17)
Germinated soybean proteins	Caco-2, HT-29, HCT-116 human colon cancer	Gonzalez-Montoya et al (13)

Six days germinated soybean protein	Cervical (HeLa, SiHa, CasKi) and breast (MCF7 and MDA-MB-231) cancer cell lines	Gonzalez-Montoya et al (33)
Vglycin	CT-26, SW480, and NCL-H716 colon cancer cells	Gao et al. (34)
Soy protein isolate hydrolysates	HSC-3 human oral cancer cells	Heish et al. (12)

3.2. Cytotoxic activity

The screening of the cytotoxic activity of the bioactive peptides of soybean extracts against PC-3 cell lines was measured using the MTT assay. The current study found no cytotoxic activities of the different samples against the cell lines when compared to untreated cells (Fig. 1 and 2).

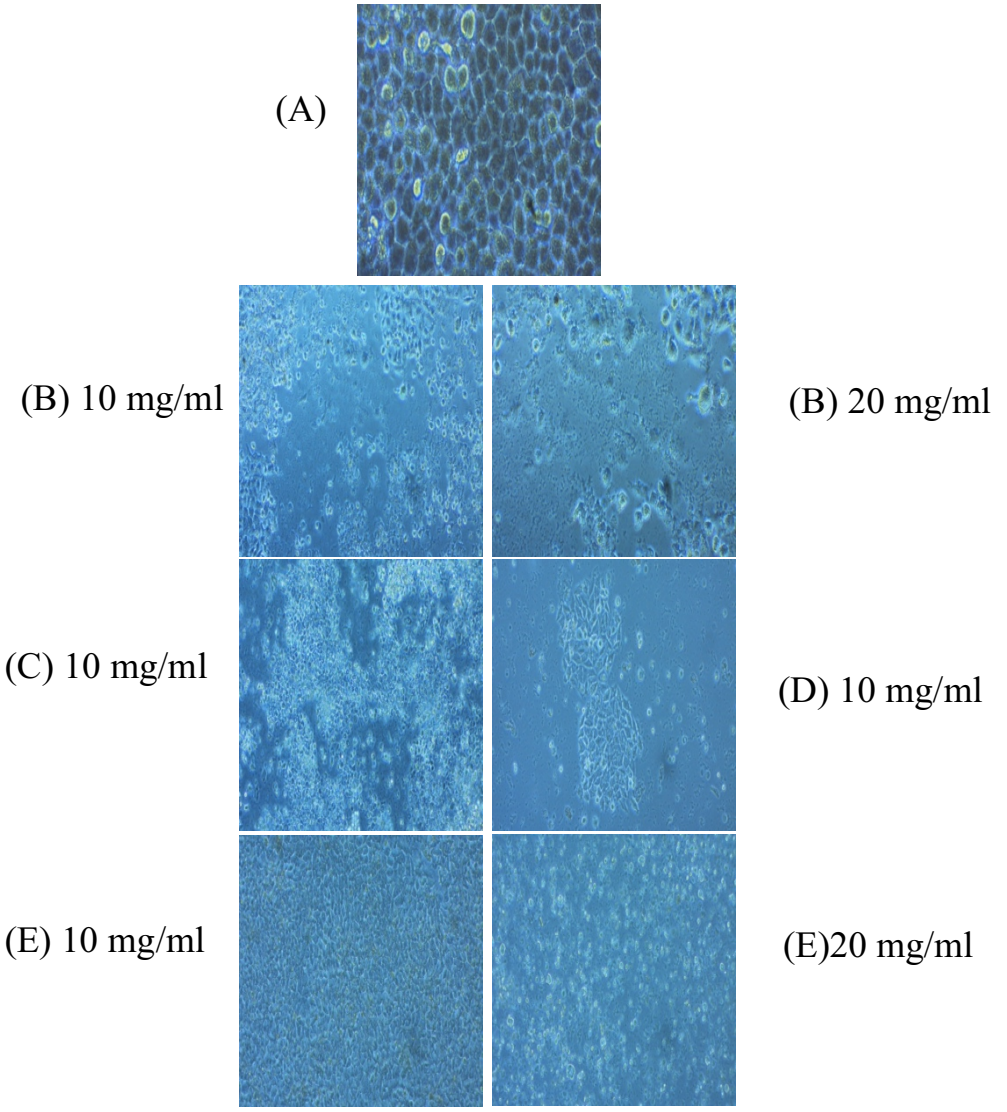

Figure 1. Effect of soybean peptides (10 and 20 mg/mL) on PC-3 cells 48 h after incubation. (A) control cell (Without peptide extract); (B) Total hydrolysate (10 and 20 mg/ml; (C) HMMH (10 mg/ml); (D) LMMH (10 mg/ml); (E) NH (10 and 20 mg/ml).

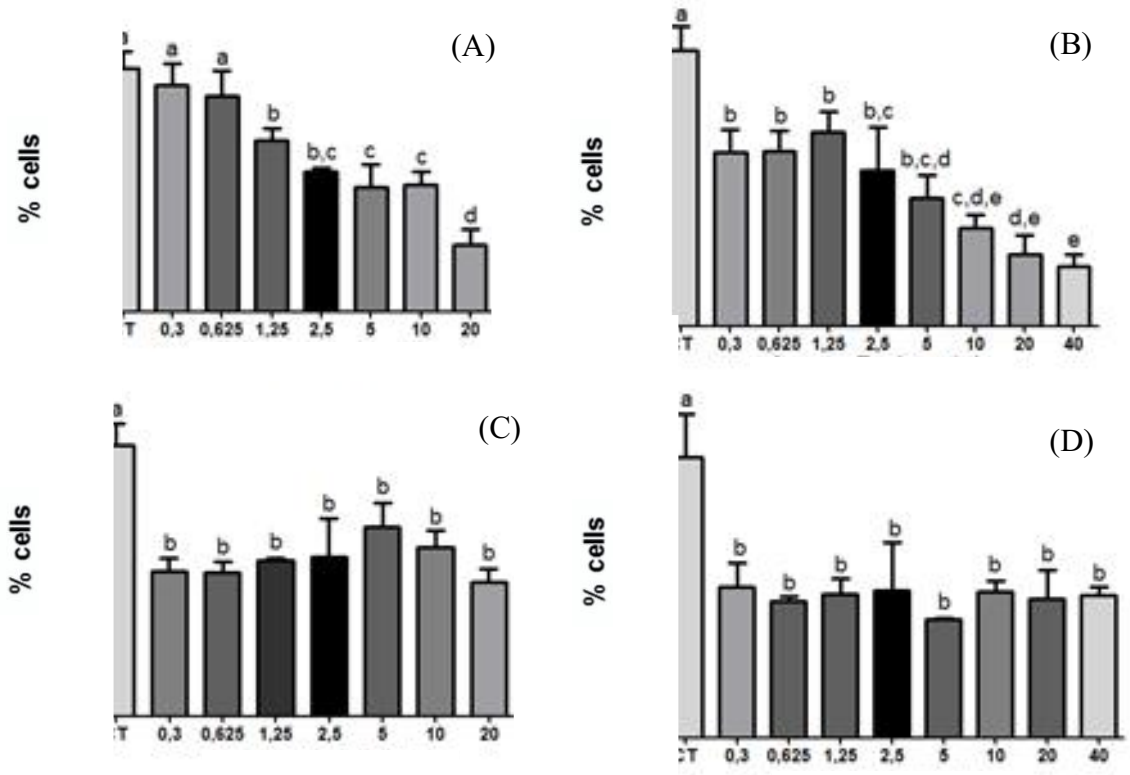

Figure 2. Effect of **(**A) NH; (B) TH; (C) LMMH; (D) HMMH on viability (mean SD) of PC-3 cells 48 h after incubation. Significant differences between the untreated cells (CT) and those incubated with the respective extracts (0,3 to 40 mg/mL) were compared by the One-way ANOVA test, with Tukey post-test (* $p < 0.05$; same letters indicated no significant difference).

No significative difference was found between the doses (0.3 to 40 mg/mL) in the samples LMMH and HMMH in PC- 3 cells. Both samples showed a minimum average inhibition of 40% among the studied concentrations and, therefore, the concentration of 10 mg/mL was chosen to further tests. The sample with the total hydrolysate promoted a reduction of cells, with an average of inhibition of 70.0% at this concentration ($p < 0.05$). The non-hydrolyzed sample caused a decrease in PC-3 viability (Fig. 2) compared to control (70.0%) at concentration of 20 mg/mL. PC-3 viability was further reduced in all samples. LMMH and HMMH caused reductions of about 40 to 50% in cell viability in all concentrations. *In vivo* studies have already demonstrated the ability of soy peptides to attenuate tumor growth in colon cancer (34-35).

Most prostate cancer tumors require androgen receptor (AR) signaling for survival. During the progression to androgen-independence, this signaling cascade has been found to be altered at many levels within prostate cancers. PC3 cells do not express AR and are androgen-independent. They show highly aggressive behavior which is unlike most clinical cases of prostate cancers. Therefore, intense investigations

are required to better understand the androgen-independent impairment and identification of the most effective therapeutic approach toward prostate cancer. Rayaprolu et al (17) hydrolyzing soybean with Alcalase and gastrointestinal enzymes obtained peptides from 5–10 kDa showing 63% inhibition against the PC-3 prostate cancer cells, while the 10–50 kDa fraction inhibited 58% of the cells. Our better result in the antiproliferative action was in the sample TH as mentioned above.

3.3. Cell cycle progression

The flow cytometry results are showed in Table 2. The cell cycle is divided into 4 phases: the G1 phase (G for gap, meaning gap) during which the cell grows due to protein synthesis, the S phase (S for synthesis) during which the cell duplicates its DNA, the G2 phase which is another growth phase and the M phase (M for mitosis) which represents the cell division itself (36). In the MTT assay, 10.0 and 20.0 mg/mL concentrations of TH and NH samples caused the greatest reduction in cell viability, with significative difference, which was greater after 48 h. Hence, these concentrations were used for this interval in the cell cycle and apoptosis assays. For the HMMH and LMMH samples only the concentration of 10.0 mg/mL was tested.

Table 2. Effect of soy protein hydrolysates (10.0 and 20.0 mg/mL) on cell cycle progression in PC-3 cells 48 h after incubation**.**

Sample	Concentration	G_O/G_1	S	G_2/M
CT	-	54.47 ± 8.45	2.20 ± 0.50	19.90 ± 4.46
TH	10 mg/ml	80.67 ± 4.22**	7.29 ± 1.23**	11.13 ± 3.25*
TH	20 mg/ml	84.30 ± 0.40**	10.38 ± 1.83**	4.64 ± 1.86**
HMMH	10 mg/ml	51.47 ± 3.06	21.00 ± 10.83**	24.17 ± 10.60
LMMH	10 mg/ml	66.30 ± 13.53*	5.77 ± 1.73**	18.73 ± 5.70
NH	10 mg/ml	87.00 ± 1.61**	9.94 ± 1.03**	2.90 ± 0.49**
NH	20 mg/ml	85.77 ± 13.75**	12.71 ± 7.62**	6.68 ± 3.87**

Results are expressed as percentage of total cells. Significant differences between untreated cells (Control) and cells treated with soybean extracts were compared (* $p < 0.05$; ** $p < 0.01$).

It was observed an increase in the percentage of PC-3 cells in G0/G1, followed by a decrease in S and G2/M phase after incubation with TH, LMMH, HMMH and NH

samples (10.0 and 20.0 mg/mL). The higher number of cells in the S phase was observed after treatment with sample HMMH at 10.0 mg/mL (21% ± 10,83) when compared to the control (Fig. 3C).

In the G0/G1 phases, HMMH at 10 mg/mL (51,47 % ± 3,06) caused a slight decrease in the percentage of cells, with no significant difference compared to the control, followed by LMMH at 10.0 mg/mL (66,30 % ± 13.53) ($p < 0.05$). Other samples caused statistical difference compared to the control with $p < 0,01$. All the samples increased cells in the S phase ($p < 0.001$). The samples HMMH and LMMH had no difference compared to the control in G2/M phases ($p < 0.001$), with 24,17 % ± 10,60 and 18,73 % ± 5,70, respectively (Fig, 3C and D). As we can see all the samples were capable of arrest the cell cycle in G0/G1 phase, and the LMMH and HMMH in the G2/M. The cell cycle arrest in G2/M phase is vital, because it leads to apoptosis when cells cannot recover and proceed to cell division.

The cell proliferation process is strictly controlled and is basically under the command of two groups of proteins, the kinases and their regulatory subunits, the cyclins. Kinases have catalytic activity and are known as cyclin-dependent kinases (CDK) due to their activation when bound to cyclins. Different cyclin-CDK complexes control cell cycle progression during the G1, S, G2 and M phases. The main cell cycle transitions are processes dependent on different cyclin-CDK complexes: the decision to enter quiescence (G0); DNA synthesis (S phase) and the transition from G2 phase to mitosis are examples of these processes (37). It has been previously reported that peptides from germinated soybean inhibited proliferation of Caco-2, HT-29, and HCT-116 human colon cancer cells in a dose-dependent manner, and peptides with higher molecular weight (> 10 kDa and 5–10 kDa fractions) are more effective at inhibiting colon cancer cell proliferation than smaller molecular weight peptides (< 5 kDa). The authors further mention that serine residues in the peptides that may be responsible for this anticancer action were identified and that they selectively induced apoptosis and mitochondrial disruption in colon cancer cells and the potential mechanistic explanation involved the inhibition of cyclin B-1 (regulatory protein predominantly expressed in the G2/M phase) and the overexpression of p21, both considered important regulators of the cell cycle, given that inside the cell, CDK2 is inhibited by the CDK inhibitor p21 (13).

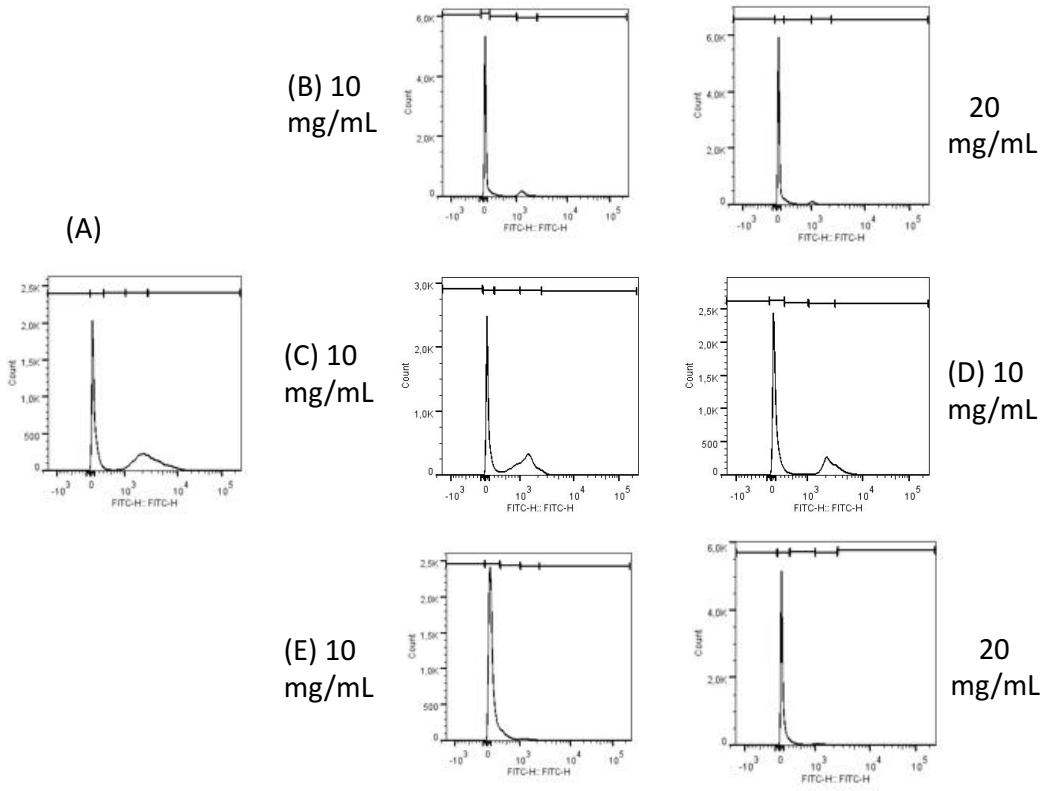

Figure 3. Illustration of effect (A) control; (B) total hydrolysate (10 and 20 mg/mL); (C) HMMH (10 mg/mL); (D) LMMH (10 mg/mL); (E) NH (10 and 20 mg/mL) on cell cycle progression in PC-3 cells 48 h after incubation. The phases of the cell cycle are illustrated at untreated cell (CT) and treated with 10.0 and 20.0 mg/mL of these hydrolysates.

Rayaprolu and coworkers (17) showed that the 5–10 kDa fraction from the soybean hydrolysated with Alcalase showed the highest reduction at 10 mg/mL on PC-3 cell counts which was not significantly different in comparison to the reduction by genistein (positive control) (P value = 0.5293). A 75% growth inhibition in Caco-2, HCT-116, and HT-29 cells was reported by a soybean 18 kDa peptide obtained after enzymatic hydrolysis. When comparing hydrolysates from soybean protein obtained either by GI-simulated digestion alone or by an alcalase hydrolysis followed by GI-simulated digestion, it was determined that when there was no alcalase previous treatment, a 7-fold higher concentration was needed to achieve the same cell growth inhibitory effect (38). Lunasin is also able to inactivate the tumor suppressor proteins, Rb, p53, and pp32, and competes with the histone acetyltransferases in binding to the

core deacetylated histones H3 and H4, and switching off the transcription, leading to arrest of the G1/S phase and causing apoptosis (39).

3.4. Apoptosis

Since cell apoptosis may be one of the consequences of cell cycle arrest, we examined if our extracts induced apoptosis in PC-3 cells. Apoptosis induction was reported by annexin V and PI biomarkers and can be triggered either by the caspase-mediated extrinsic or intrinsic pathways. As for the intrinsic pathway, several proteins are involved: the protein p53 promotes the activation of Bax, which becomes mitochondria membrane-bound, allowing cytochrome C release. This molecule promotes the activation of caspase-9, which in turn will activate the executioner caspase-3, responsible for apoptotic hallmarks such as plasma membrane asymmetry, chromatin condensation, and DNA disruption (39). We examined the effect of soybean hydrolysates on diferente stages of the PC-3 cell death process for 48 h. Table 3 shows the percentages of viable, early apoptotic, late apoptotic, and non-apoptotic cells after treatment with soybean hydrolysates (10 and 20 mg/mL) and Figure 4 shows the influence of control and the samples on the apoptosis rate.

There was no significant difference in the percentage of viable cells in relation to the control in the samples HMMH (96.50% ± 0.42) and NH (89.04 % ± 10.28 at 10 mg/mL; 83.48 % ± 18.52 at 20 mg/mL). The samples TH and NH both at 20 mg/mL had the highest value with statistical difference in early apoptosis (6.17 % ± 3.21 and 6.39 % ± 3.10, respectively) and the smallest value was HMMH (0.35 % ± 0.18) compared to the control (2.60 % ± 0.20). In the case of non-apoptotic cell death remained almost unchanged, only TH (20 mg/mL) and LMMH had difference compared to the control ($p < 0.01$), possibly indicating low toxicity in the extracts. The percentages of late apoptotic cells increased only in those PC-3 cells treated with higher concentrations of extracts in TH (20 mg/mL) to 25,2 % and LMMH (10 mg/mL) to 18,57 % (Figure 4), speculating these extracts to induce apoptosis.

Table 3. Effect of soybean peptides (10.0 mg/mL and 20.0 mg/mL) on stages of death process in human prostate adenocarcinoma cells (PC-3) after 48 h.

Samples	Concentration	Viable cells	Early apoptosis	Late apoptosis	Nonapoptotic Cell Death
Control	-	94,13 ± 2.34[a]	2.60 ± 0.20[a]	5.93 ± 3.13[a]	0.40 ± 0.23[a]
TH	10 mg/ml	77.60 ± 10.64[b]	0.71 ± 0.43[a]	16.10 ± 5.70[b]	0.93 ± 0.42[a]
TH	20 mg/ml	76.80 ± 9.54[b]	6.17 ± 3.21[b]	25.20 ± 7.96[b]	1.03 ± 0.47[b]
HMMH	10 mg/ml	96.50 ± 0.42[a]	0.35 ± 0.18[c]	3.30 ± 0.28[a]	0.46 ± 0.37[a]
LMMH	10 mg/ml	75.73 ± 6.52[b]	3.03 ± 2.46[a]	18.57 ± 7.45[b]	1.82 ± 0.42[b]
NH	10 mg/ml	89.04 ± 10.28[a]	4.39 ± 2.68[a]	2.86 ± 1.60[a]	0.64 ± 0.46[a]
NH	20 mg/ml	83.48 ± 18.52[a]	6.39 ± 3.10[b]	3.56 ± 2.50[a]	0.55 ± 0.35[a]

Results are expressed as percentage of total cells. Different letters represented significant differences between untreated cells (Control) and cells treated with soybean extracts were compared ($p < 0.05$).

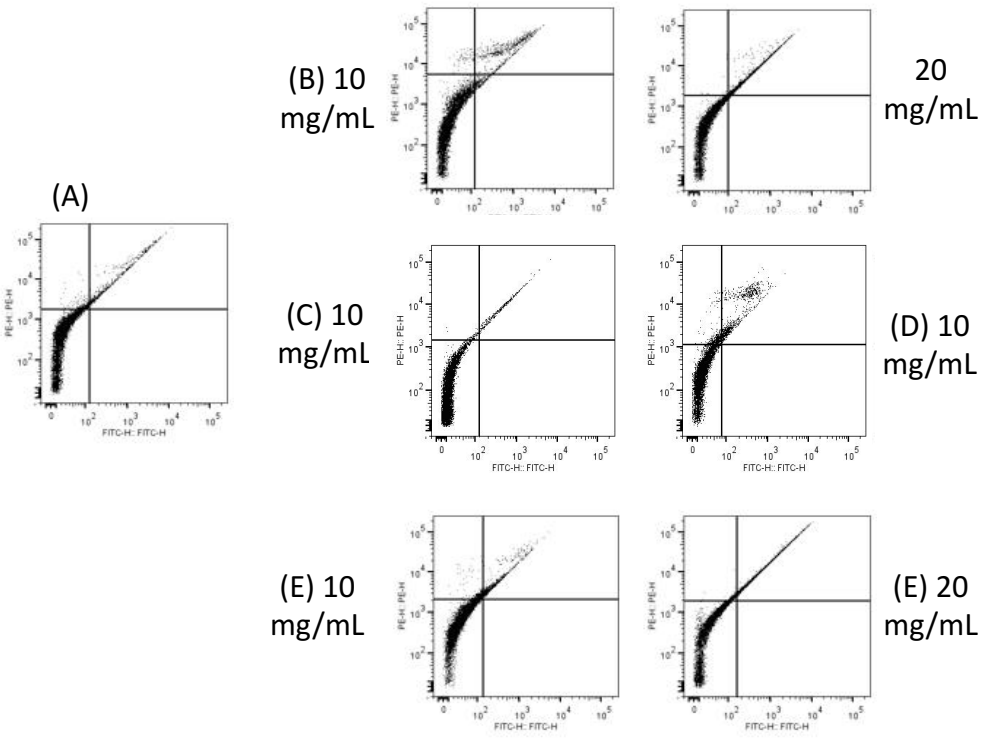

Figure 4. Apoptosis induction in 48 h in PC-3. Illustration of effect (A) control; (B) total hydrolysate (10 and 20 mg/mL); (C) HMMH (10 mg/mL); (D) LMMH (10 mg/mL); (E) NH (10 and 20 mg/mL) on rate of apoptosis PC-3 cells 48 h after incubation according to concentration of these samples. Untreated cell (CT) and treated with 10.0 and 20.0 mg/mL.

Gonzalez-Montoya et al. (33) showed that peptides from six days germinated soybean hydrolysed demonstrated an interesting antiproliferative activity on breast and cervical cancer cell lines, as well as the ability of inducing apoptosis with minimal effects on normal cells, the fraction H6 and >10 kDa peptides also showed notable antioxidant activity, they attributed these two activities to the large amount of hydrophobic amino acids found in the two fractions. The initial association of peptides with the cancer cell membrane occurs through electrostatic interactions between the cationic peptide and anionic lipopolysaccharide in the outer membrane leading to membrane perturbation. The net charge and the number of positive charge also influence the activity of peptides (16).

Soy-derived peptides have already been reported in the literature as anticancer in colon cell lines, such as Vglycine, a 37-amino acid peptide, which according to Gao et al. (34) promotes apoptosis through activation of mitochondrial pathways, as evidenced by down-regulation of the Bcl-2 expression, up-regulation of the Bax expression, alteration of the Bcl-2/Bax rate, and activation of the caspase-3 activity. Outro estudo recente com hidrolisado proteico de soja por thermolisyn na concentracao de 1 mg/mL indicou que esse extrato foi capaz de induzir a apoptose das células de cancer oral (HSC-3) por meio da regulação positiva do modulador da apoptose, p53, da regulação negativa do inibidor da apoptose, Bcl-2, e então ativando a família de proteínas relacionadas à caspases (12).

In vivo experiments and clinical trials are needed to demonstrate the physiological effect of peptides, but *in vitro* studies remain important prospective tools because peptide functionality is based on biological mechanisms. They cannot, however, replace *in vivo* and clinical studies because it is very difficult to establish a direct relationship between *in vitro* and *in vivo* biological activity. Peptide bioavailability after oral administration is one of the main reasons for this incomparability and one of the primary aspects to study before bioactive peptides can be incorporated into food or drug systems (3).

3. Conclusion

These data show that hydrolysates of soybean were able to inhibit cell proliferation, to arrest cell cycle in different phases in the tested prostate cancer cell line (PC-3). As we could see in cell cycle analysis the TH without fractionation (10 mg/mL)

had the better capacity to arrest the cell cycle, followed by the non-hydrolysed sample (20 mg/mL). Regarding apoptosis, TH indicated a higher apoptosis index at a concentration of 20 mg/ml, followed by the fraction with peptides smaller than 3 kDa (LMMH). Our data suggest that soybean extracts, especially TH exhibit anticancer activity against prostate cancer cells by causing apoptosis.

However, further cell experiments are needed to confirm these findings and to evaluate the molecular mechanisms, estructure and pharmacokinetics involved in the effects observed. In addition, *in vivo* and clinical tests would be required to allow the recommendation of consuming of these types of peptides to help protect against prostate cancer. Bioavailability and bioaccessibility of extracts should be investigated to determine the quantity needed to be ingest to achieve such effects, due to possible losses during digestion, absorption and metabolization by gut microbiota.

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CONSIDERAÇÕES FINAIS

O presente estudo forneceu informações sobre os peptideos gerados a partir de concentrado proteico de soja hidrolisados com papaína e alguns de seus possíveis efeitos biológicos *in vitro*. Foram avaliadas as atividades antioxidante, antidiabética e anticancerigenas desses peptídeos. Também foi realizado a identificação de peptídeos bioativos por espectometria de massas e com isso a comparação com a digestão *in silico* foi obtida.

A fração de alta massa molecular (HMMH) foi capaz de retardar a oxidação lipídica nas misturas de sardinha sob refrigeração, além de inibir a ação da α-glicosidase (24%) e da lipase pancreática (30%). A fração de menor massa molecular (LMMH), embora não indicada como antioxidante, mostrou potencial para inibir α-glicosidases (36%) e lipases (30%). A avaliação da bioatividade *in silico* correlacionou-se mal com os testes antioxidantes *in vitro*, mas bem com a inibição da α-glicosidase *in vitro*.

Os hidrolisados de soja foram capazes de inibir a proliferação celular e interromper o ciclo celular em diferentes fases na linhagem celular de câncer de próstata testada (PC-3). A amostra TH sem fracionamento (10 mg/mL) teve a melhor capacidade de interromper o ciclo celular, seguido pela amostra não hidrolisada (20 mg/mL). Em relação à apoptose, o NH indicou maior índice de apoptose na concentração de 20 mg/ml, seguido da fração com peptídeos menores que 3 kDa (LMMH).

Em geral, a produção de peptídeos com a enzima de origem vegetal papaína se mostrou eficiente não apenas na obtenção de peptídeos antioxidantes mas com outras atividades. Como planos futuros desse estudo esta entender o mecanismo de ação, biodisponibilidade e bioacessibilidade envolvidos nessas atividades e a purificação das sequencias encontradas para possível uso em alimentos.

ANEXOS

- Capitulo II

Supplementary Table 1. Selected studies without peptide sequence and their respective outcomes of interest.

Supplementary data 2. Studies that were selected and evaluated in the bibliometric review

- Capitulo III

Supplementary Data S1 – Comparison between sequences found by mass spectrum and sequences found by in silico simulated digestion.

Supplementary Data S2 - Structure–activity relationships of soy-derived antioxidant and alpha-glucosidase inhibitor peptides identified by MS.

- Capitulo IV

Supplementary Table 1 – Peptides of soybean hydrolysate identified by Mass Spectometry. Bold and red sequences represented peptides that have anticancer activity by BIOPEP-UWM

SUPPLEMENTARY MATERIAL

CAPÍTULO II

Critical Review for the Production of Antidiabetic Peptides by a Bibliometric Approach

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Supplementary Table 1. Selected studies without peptide sequence and their respective outcomes of interest.

Authors (year)	Source of protein	Peptide Sequence	Outcomes of interest
Han, et al. [13]	Oilseed and dairy protein hydrolysates	_____	Oilseed proteins could be considered as comparable sources of ACE and alpha-glucosidase inhibitory peptides, especially soybean.
Kong, et al. [58]	Walnut (Juglans regia L.)	Table 1A	The peptide fraction with lower molecular weight and higher basic amino acid residues possessed strong DPP-IV inhibitory activity. Nine novel effective bioactive peptides were identified in the fraction.
Mazloomi, et al. [65]	Orange seed	_____	Could be used as a health-promoting ingredient to help in the reduction of blood pressure and the regulation of diabetes.
Rivero-Pino, et al. [19]	Tenebrio molitor	Table 3 and Table 4	Potential ingredients in functional foods intended for the regulation of diabetes.
Acquah, et al. [1]	Review	Table 2	Bioactive peptides could serve as important primary strategies for management and/or control of diabetes.
Akan, E. [28]	Camel milk and donkey milk	_____	Peptides showed better antidiabetic and antioxidant activity compared to whey-derived peptides.
Harnedy-Rothwell, et al. [66]	Boarfish (Capros aper)	Table 3	IPVDM, a potent DPP-IV inhibitory activity in the in vitro assay, also showed potent activity in a cell-based DPP-IV inhibitory assay.

Supplementary Table 1. Selected studies without peptide sequence and their respective outcomes of interest.

Authors (year)	Source of protein	Peptide Sequence	Outcomes of interest
Karimi, et al. [17]	Corn germ protein	_____	The fractions showed high radical scavenging and α-glucosidase inhibitory activity, and α-amylase inhibitory activity can be attributed to high levels of hydrophobic amino acids.
Kehinde and Sharma [59]	Review	All tables	Several BHs and BPs have been isolated from dairy, meat, cereals and legumes;
Li, et al. [67]	Arthrospira Platensis (Spirulina)	_____	Tryptic phycobiliproteins hydrolysate is a new source of peptides for the development of nutraceuticals or functional foods.
Megrous, et al. [12]	Casein Hydrolysates	_____	Casein hydrolysates generated by metalloendopeptidase under selected hydrolysis conditions showed significant antidiabetic properties.
Mudgil, et al. [25]	Quinoa (Chenopodium quinoa Willd.)	Table 2	Effective inhibitory properties towards enzymatic biomarkers of diabetes (DPP-IV and AG) and hypertension (ACE).
Ohara, et al. [24]	Common bean (Phaseolus vulgaris L. cv Carioca)	_____	Antidiabetic potential was evidenced by total inhibition of alpha-amylase activity and reduction of alpha-glucosidase activity by 34.73%.
Olagunju, et al. [68]	Pigeon pea (Cajanus cajan)	_____	Potential ingredients to formulate antihypertensive and antidiabetic functional foods and nutraceuticals.

Supplementary Table 1. Selected studies without peptide sequence and their respective outcomes of interest.

Authors (year)	Source of protein	Peptide Sequence	Outcomes of interest
Patil, et al. [5]	Review	Table 2	Bioactive peptides inhibit enzymes such as alpha-glucosidase, alpha-amylase, dipeptidyl peptidase-IV and glucose transporter systems involved in type 2 diabetes.
Rivero-Pino, et al. [11]	Sardine pilchardus	Table 3	The most bioactive fraction ranges from 800 to 1400 Da.
Rivero-Pino, et al. [18]	Review	Table 2	The potential of bioactive peptides as antidiabetic agents to be employed in food formulation is a relevant field of research.
Rivero-Pino, et al. [69]	Tenebrio molitor	———	Ultrasound pre-treatment modifies the native structure of the protein and subtilisin hydrolysis reduces the size of the peptide chain.
Wu, et al. [6]	Review	Review of peptides from cereals and pseudo cereals	Protein hydrolysates and peptides isolated from rice, wheat, oats, buckwheat, quinoa, barley, and corn have antidiabetic effects.
Yap, et al. [70]	Review	Table 1	Bioinformatics approach emerges as an innovative breakthrough to ameliorate the time and economic viability of traditional.
Zamudio and Campos [71]	Review	Review of proteins and peptides from animal and plant sources focused on amaranth, quinoa and chia	Excellent alternative for further development of antidiabetic functional food and nutraceuticals.

Supplementary Table 1. Selected studies without peptide sequence and their respective outcomes of interest.

Authors (year)	Source of protein	Peptide Sequence	Outcomes of interest
Casanova-Martí, et al. [57]	Chicken feet (Gallus gallus domesticus)	_____	Was a good source of DPP-IV inhibitors reducing glycaemia in glucose-intolerant rats and provided good stimulation of endogenous GLP-1 secretion.
Cermeño, et al. [22]	Porphyra dioica	Table 4	Peptides therein may be used as multifunctional ingredients in nutraceutical or functional food products.
Connolly, et al. [72]	Brewers' spent grain	_____	The extraction of bioactive peptides from wet BSG by direct hydrolysis is a viable method for processing BSG.
Gomez, et al. [21]	Portuguese Oyster (Crassostrea angulata)	_____	Good source of peptides with ACE and DPP-IV inhibitory activities.
Ibrahim, et al. [73]	In silico analysis	Table 2	Potato-derived BP: antidiabetic and antimicrobial potentials; yam-derived BP: antihypertensive and anticancer agents.
Kęska, et al. [74]	Porcine (Sus scrofa) skeletal muscle	Table 4	Digested *in silico* by gastrointestinal enzymes have a high potential for the management of blood glucose levels in patients with T2DM.
Lima, et al. [75]	Chicken by-product	Table 2	Can potentially serve as ingredients of multi-functional foods with dual effects of DPP-IV inhibition and enhancement of cellular glucose uptake.

Supplementary Table 1. Selected studies without peptide sequence and their respective outcomes of interest.

Authors (year)	Source of protein	Peptide Sequence	Outcomes of interest
Liu, et al. [64]	Review	Table 2	Diets rich in specific bioactive ingredients, including food protein-derived peptides, have potential application in the prevention and management of T2DM.
Mudgil, et al. [37]	Camel skin (Camelius dromedaries)	_____	ACE, DPP-IV and pancreatic α-amylase inhibition by camel skin gelatin hydrolysates was reported for the first time.
Park and Yoon, [40]	Perilla (Perilla frutescens var. japonica Hara)	_____	Peptides fractionated by UF were found to have various physiological functions: antioxidant, antidiabetic and antihypertensive activities.
Valencia-Mejía, et al. [32]	Beans (Phaseolus vulgaris L.)	_____	Hydrolysis of common beans was able to produce molecules with higher hypoglycemic and antihyperglycemic activities.
Yan, et al. [3]	Review	Table 1	Bioactive peptides, particularly from natural products, show high potential for application in the management and treatment of diabetes.
González-Montoya, et al. [20]	Soybean protein	Table 2	First report. Inhibition of DPP-IV, α-amylase and intestinal α-glucosidases with potential antidiabetic properties.
Hall, et al. [34]	Cricket (Gryllodes sigillatus)	_____	Peptides displayed good ACE, DPP-IV inhibition, and antioxidant activity; bioactivity increased, in most cases, after simulated gastrointestinal digestion.

Supplementary Table 1. Selected studies without peptide sequence and their respective outcomes of interest.

Authors (year)	Source of protein	Peptide Sequence	Outcomes of interest
Harnedy, et al. [16]	Atlantic salmon (Salmo salar)	_____	Hydrolysates/peptides with significant antidiabetic (insulin and GLP-1 secretory and DPP-IV inhibitory) activity in vitro.
Mudgil, et al. [63]	Camel milk (Camelius dromedaries)	_____	Camel milk protein hydrolysates effectively inhibited DPP-IV, lipase and α-amylase.
Nongonierma, et al. [35]	Cricket (Gryllodes sigillatus)	_____	First time report. The CP contains endogenous enzymes which were able per se to hydrolyze *G. sigillatus* proteins and yield samples with DPP-IV inhibitory properties.
Wang, et al. [56]	Walnuts (Juglans mandshurica Maxim.)	_____	*In vivo*: WHPs alleviated insulin resistance by increasing insulin secretion, and liver GK and glycogen levels as well as by decreasing fasting blood glucose level.
Neves, et al. [27]	Atlantic salmon (Salmo salar)	Table 2	Peptides have biological activities, such as ACE and DPP-IV inhibition, and antioxidant capacity.
Nongonierma, et al. [76]	Camel milk (Camelus dromedarius)	Table 2 and Table 4	The first time that camel milk proteins serve as an interesting source of DPP-IV inhibitory peptides by using an *in silico* analysis.
Nongonierma, et al. [77]	Milk protein isolate	Table 3 and Table 4	Some of these sequences were previously reported to be DPP-IV inhibitors or to possess structural features of DPP-IV inhibitory peptides.

Supplementary Table 1. Selected studies without peptide sequence and their respective outcomes of interest.

Authors (year)	Source of protein	Peptide Sequence	Outcomes of interest
Nongonierma, et al. [78]	Wheat gluten	Table 4	Hydrolysate contained short (di- and tri-) peptides previously identified as DPP-IV inhibitors, and several peptides possessing DPP-IV inhibitory features.
Nongonierma, et al. [2]	Review	Table 1	Potent DPP-IV inhibitory peptides have been identified in several food protein-derived hydrolysates.
Nongonierma, et al. [79]	Bovine milk protein	Table 3 and Table 4	Several known, potent DPP-IV inhibitory peptides were identified within the milk protein hydrolysates.
Xia, et al. [33]	Review	Table 2	Natural peptides derived from several kinds of marine organisms showed great potential to regulate glucose metabolism for insulin-resistant individuals.
Mojica and Meíja, [31]	Common beans (Phaseolus vulgaris L.)	Table 2	Alcalase protein fractions showed outstanding antidiabetic potential by inhibiting the targeted enzymes through hydrogen bonds, polar and hydrophobic interactions.
Nongonierma, et al. [23]	Casein hydrolysates	———	A multi-functional hydrolysate, H12 (pH 8.0, 40 ∘C and 5 h), yielded high DPP-IV inhibitory and antioxidant activities.
Siow and Gan, [80]	Cumin seeds (Cuminum Cyminum)	———	Antioxidant activity may turn them into potential ingredients of health-promoting or functional foods.

Supplementary Table 1. Selected studies without peptide sequence and their respective outcomes of interest.

Authors (year)	Source of protein	Peptide Sequence	Outcomes of interest
Uraipong & Zhao, [62]	Rice bran (cultivar Reiziq)	_____	α-amylase, α-glucosidase and ACE-inhibition activities comparable in magnitude to acarbose.

Supplementary data 2. Studies that were selected and evaluated in the bibliometric review

Authors (year)	Country	Type of inhibition	Type of analysis	Type of document	Journal	Citations
Han et al. [13]	United Kingdom	α-glucosidase, DPP-IV	*in vitro*	Original	Current Research in Food Science	0
Kong et al. [58]	China	DPP-IV	*in vitro*	Original	Food Chemistry	0
Mazloomi et al. [65]	Spain, Iran	α-glucosidase, α-amylase	*in vitro*	Original	Foods	0
Mudgil et al. [42]	United Arab Emirates, Malaysia	α-amylase, α-glucosidase, DPP-IV	*in vitro, in silico*	Original	Food Chemistry	2
Rivero-Pino et al. [19]	Spain	DPP-IV	*in vitro, in silico*	Original	Food Chemistry	0
Rivero-Pino et al. [81]	Spain	DPP-IV, α-glucosidase	*in vitro, in silico*	Original	Food & Function	1
Acquah et al. [1]	Canada, Australia, New Zealand	α-amylase, α-glucosidase	*in vitro and in vivo*	Review	Critical Reviews in Food Science and Nutrition	1
Akan [28]	Turkey	α-glucosidase, DPP-IV	*in vitro*	Original	Journal of Food Science and Technology	1
Feng et al. [43]	China	α-glucosidase	*in vitro*	Original	International Journal of Food Science and Technology	1
Gao et al. [44]	China	DPP-IV	*in vitro*	Original	Molecules	0
Harnedy-Rothwell et al. [66]	Ireland, United Kingdom	DPP-IV	*in vitro, in situ*	Original	Food Research International	2
Ibrahim et al. [38]	South Africa, Nigeria	DPP-IV, α-glucosidase	*in vitro, in silico*	Original	International Journal of Peptide Research and Therapeutics	0
Jia et al. [45]	China	DPP-IV	*in vitro*	Original	Food Chemistry	6
Jin et al. [10]	China	DPP-IV	*in vitro*	Original	Food Research International	2
Karimi et al. [17]	Iran	α-glucosidase, α-amylase, DPP-IV	*in vitro*	Original	Food Science and Nutrition	6

Kehinde and Sharma [59]	India	DPP-IV	-	Review	Critical Reviews in Food Science and Nutrition	17
Li et al. [67]	Italy	DPP-IV	*in vitro*	Original	Nutrients	2
Megrous et al. [12]	China	α-glucosidase, α-amylase	*in vitro*	Original	International Journal of Peptide Research and Therapeutics	0
Mudgil et al. [25]	United Arab Emirates, Malaysia, Ireland	DPP-IV	*in vitro, in silico*	Original	Journal of Cereal Science	1
Ohara et al. [24]	Brazil	α-amylase, α-glucosidase	*in vitro*	Original	Biocatalysts and Biotransformation	5
Olagunju et al. [68]	Nigeria, Canada	α-amylase, α-glucosidase	*in vitro*	Original	Journal of Food Biochemistry	0
Patil et al. [5]	India	α-glucosidase, α-amylase, DPP–IV	*in vitro, in vivo*	Review	International Journal of Peptide Research and Therapeutics	9
Rivero-Pino et al. [11]	Spain	DPP-IV	*in vitro, in silico*	Original	Food Chemistry	42
Rivero-Pino et al. [18]	Spain	--	*in vitro, in silico*	Review	Foods	7
Rivero-Pino et al. [69]	Spain	α-glucosidase	*in vitro*	Original	Food and Bioproducts Processing	2
Wu et al. [6]	China	--	*in vitro and in vivo*	Review	E3S Web of Conferences	0
Yap et al. [70]	Malaysia	DPP-IV, α-amylase, α-glucosidase	*in silico, in vivo*	Review	Trends in Food Science and Technology	2
Zamudio and Campos [71]	Mexico	α-amylase, α-glucosidase, DPP-IV	*in vitro and in vivo*	Review	Critical Reviews in Food Science and Nutrition	0
Casanova-Martí et al. [57]	Spain	DPP-IV	*in vitro, in vivo*	Original	Food & Function	6
Cermeño et al. [22]	Ireland	DPP-IV	*in vitro*	Original	Food & Function	5
Connolly et al. [72]	Ireland	DPP-IV	*in vitro*	Original	Food Research International	18

Gomez et al. [21]	Philippines, Taiwan	DPP-IV	*in vitro, in silico*	Original	International Journal of Molecular Sciences	4
Ibrahim et al. [73]	South Africa, Nigeria	DPP-IV	*in silico*	Original	International Journal of Peptide Research and Therapeutics	6
Kęska et al. [74]	Poland	DPP-IV	*in silico*	Original	Nutrients	2
Lima et al. [75]	Denmark	DPP-IV	*in vitro*	Original	Food & Function	2
Liu et al. [64]	China, USA	DPP-IV	*in silico and in vivo*	Review	International Journal of Molecular Sciences	47
Mudgil et al. [37]	United Arab Emirates	α-amylase, DPP-IV	*in vitro*	Original	LWT	10
Nongonierma et al. [46]	Ireland, United Arab Emirates	DPP-IV	*in silico and in silico*	Original	Food Chemistry	4
Park and Yoon [40]	Korea	α-amylase, α-glucosidase	*in vitro*	Original	Czech Journal of Food Sciences	6
Valencia-Mejía et al. [32]	Brazil, Mexico	α-amylase, α-glucosidase	*in vitro, in vivo*	Original	Food Research International	5
Vilcacundo et al. [47]	Spain, Ecuador	DPP-IV, α-amylase	*in vitro*	Original	Journal of the Science of Food and Agriculture	7
Wang et al. [48]	China, United Kingdom	α-glucosidase, DPP-IV	*in vitro*	Original	Food Science and Nutrition	26
Xu et al. [49]	China	DPP-IV	*in vitro*	Original	Journal of Agricultural and Food Chemistry	3
Yan et al. [3]	China	α-amylase, α-glucosidase, DPP-IV	*in vitro*	Review	International Journal of Food Science and Technology	13
Zheng et al. [50]	China	DPP-IV	*in vitro*	Original	Journal of Agricultural and Food Chemistry	1
González-Montoya et al. [20]	Mexico and Spain	DPP-IV, α-amylase, α-glucosidases	*in vitro*	Original	International Journal of Molecular Sciences	31
Hall et al. [34]	USA	DPP-IV	*in vitro*	Original	Food Chemistry	13

Harnedy et al. [16]	Ireland	DPP-IV	*in vitro*	Original	Food Research International	6
Ibrahim et al. [51]	South Africa, Nigeria	α-glucosidase, α-amylase	*in vitro, in silico*	Original	Biomedicine & Pharmacotherapy	22
Mudgil et al. [63]	United Arab Emirates, Malaysia	DPP-IV, α-glucosidase	*in vitro*	Original	Food Chemistry	57
Mune et al. [15]	Cameroon, Germany	DPP-IV	*in vitro, in silico*	Original	Food Chemistry	26
Nongonierma et al. [35]	Ireland	DPP-IV	*in vitro*	Original	Food & Function	2
Nongonierma et al. [39]	Ireland, United Arab Emirates	DPP-IV	*in vitro, in silico*	Original	Food Chemistry	9
Wang et al. [56]	China	α-glucosidase	*in vitro, in situ, in vivo*	Original	Journal of Food Biochemistry	14
Ji et al. [29]	China	DPP-IV	*in vitro*	Original	Journal of Chromatography B	6
Ji et al. [30]	China	DPP-IV	*in vitro*	Original	Journal of Food Science	1
Liu et al. [52]	China	DPP-IV	*in vitro, in silico*	Original	Molecules	4
Mojica et al. [36]	USA, Mexico	DPP-IV and α-glucosidase	*in vitro*	Original	Journal of the Science of Food and Agriculture	32
Neves et al. [27]	Ireland	DPP-IV	*in vitro*	Original	Food Chemistry	17
Nongonierma et al. [76]	Ireland, United Arab Emirates	DPP-IV	*in vitro, in silico*	Original	Journal of Functional Foods	43
Nongonierma et al. [77]	Ireland	DPP-IV	*in vitro*	Original	Food Chemistry	4
Nongonierma et al. [78]	Ireland	DPP-IV	*in vitro*	Original	Food & Function	2
Nongonierma and FitzGerald [2]	Ireland	DPP-IV	-	Review	Journal of Food Biochemistry	39
Nongonierma et al. [79]	Ireland	DPP-IV	*in vitro*	Original	Food Research International	8
Song et al. [82]	China	DPP-IV	*in vitro*	Original	Journal of Dairy Science	31

Taga et al. [53]	Japan	DPP-IV	*in vitro*	Original	Bioscience, Biotechnology, and Biochemistry	2
Uraipong and Zhao [41]	Australia	α-glucosidase	*in vitro*	Original	Journal of the Science of Food and Agriculture	20
Vilcacundo et al. [26]	Spain, Ecuador	DPP-IV, α-amylase and α-glucosidase	*in vitro*	Original	Journal of Functional Foods	67
Xia et al. [33]	China	DPP-IV, α-amylase, α-glucosidase	*in vitro, in silico, in vivo*	Review	Marine Drugs	27
Lammi et al. [54]	Italy	DPP-IV	*in vitro, in silico*	Original	Journal of Agricultural and Food Chemistry	9
Mojica and Mejía [31]	USA	DPP-IV, α-amylase and α-glucosidase	*in vitro, and in silico*	Original	Food & Function	12
Nongonierma et al. [23]	Ireland	DPP-IV	*in vitro*	Original	Journal of the Science of Food and Agriculture	19
Nongonierma et al. [55]	Ireland	DPP-IV	*in vitro, in silico*	Original	Food &Function	4
Siow and Gan [80]	Malaysia	α-amylase	*in vitro*	Original	Journal of Food Biochemistry	13
Uraipong and Zhao [62]	Australia	α-amylase and α-glucosidase	*in vitro*	Original	Journal of the Science of Food and Agriculture	61

SUPPLEMENTARY MATERIAL

CAPÍTULO III

BIOACTIVE PROPERTIES OF PEPTIDE FRACTIONS FROM BRAZILIAN SOY PROTEIN HYDROLYSATES: *IN SILICO* EVALUATION AND EXPERIMENTAL EVIDENCE

Enviado para Food Hydrocolloids in Health em 21 de Setembro de 2022.
Manuscript Number: FHFH-D-22-00163

Supplementary Data S1 – Comparison between sequences found by mass spectrum and sequences found by *in silico* simulated digestion.

	In Vitro Sequences (HMMH)	In silico Sequences (HMMH)	Cosine value	Sequence Difference	Protein
1	DSYNLQSGDALRVPAGTTYYVVNPDNDENLR	LQSGDALRVPSGTTYYVVNPDNNENLR	0.9686	0,04	P0DO15
	DSYNLQSGDALRVPAGTTYYVVNPDNDENLR	LQSGDALRVPSGTTYYVVNPDNNENLR	0.9686	0,04	P0DO16
	DSYRLQSGDALRVPSGTTYYVVNPDNNENLR	LQSGDALRVPSGTTYYVVNPDNNENLR	0.9821	0,04	P0DO15
	DSYRLQSGDALRVPSGTTYYVVNPDNNENLR	LQSGDALRVPSGTTYYVVNPDNNENLR	0.9821	0,04	P0DO16
2	EDENNPFYLRSSNSFQTLFENQNGR	SSNSFQTLFENQNGRIR	0.9157	0,08	F7J077
	EDENNPFYLRSSNSFQTLFENQNGR	SSNSFQTLFENQNGRIR	0.9157	0,08	P25974
	EDENNPFYLRSSNSFQTLFENQNGR	SSNSFQTLFENQNGRIR	0.9157	0,08	Q50JD8
3	FPLLVLLGTVFLASVCVSLKVREDENNPFYFR	FPLLLLGVVFLASVSVSFGIAYWEKQNPSHNKCLR	0.9187	0,03	P11827
	FPLLVLLGTVFLASVCVSLKVREDENNPFYFR	FPLLLLGVVFLASVSVSFGIAYWEKQNPSHNKCLR	0.9187	0,03	P11827
4	FQTLFKNQHGHLRVLQRFDQRSPQLENLR	NQYGHVRVLQRFNKRSQQLQNLR	0.9086	0,06	P11827
	FQTLFKNQHGHLRVLQRFDQRSPQLENLR	NQYGHVRVLQRFNKRSQQLQNLR	0.9086	0,06	P11827
5	LNALKPDNRIESEGGFIETWNPNNKPFQCAGVALSR	PDNRIESEGGFIETWNPNNKPFQCAGVALSRCTLNRNALR	0.9702	0,04	P04405
	LNALKPDNRIESEGGFIETWNPNNKPFQCAGVALSR	PDNRIESEGGFIETWNPNNKPFQCAGVALSRCTLNRNALR	0.9702	0,04	P11828
	LNALKPDNRIESEGGFIETWNPNNKPFQCAGVALSR	PDNRIESEGGLIETWNPNNKPFQCAGVALSRCTLNRNALR	0.9638	0,04	P04776
	LNALKPDNRIESEGGFIETWNPNNKPFQCAGVALSR	TNDRPSIGNLAGANSLLNALPEEVIQHTFNLK	0.9252	0,04	P04405
	LNALKPDNRIESEGGFIETWNPNNKPFQCAGVALSR	TNDRPSIGNLAGANSLLNALPEEVIQQTFNLR	0.9158	0,04	P11828
6	LSAEFGSLRKNAMFVPHYNLNANSIIYALNGR	TNDRPSIGNLAGANSLLNALPEEVIQHTFNLK	0.9012	0	P04405
7	PFNLGSRDPIYSNKLGKLFEITQRNPQLR	SRDPIYSNKLGKLFEITPEKNPQLR	0.9455	0,04	P11827
	PFNLGSRDPIYSNKLGKLFEITQRNPQLR	SRDPIYSNKLGKFFEITPEKNPQLR	0.9306	0,04	P0DO15
	PFNLGSRDPIYSNKLGKLFEITQRNPQLR	SRDPIYSNKLGKFFEITPEKNPQLR	0.9306	0,04	P0DO16
8	PFNLRSRDPIYSNKLGKFFEITPEK	SRDPIYSNKLGKFFEITPEKNPQLR	0.9818	0	P0DO15
	PFNLRSRDPIYSNKLGKFFEITPEK	SRDPIYSNKLGKFFEITPEKNPQLR	0.9818	0	P0DO16
	PFNLRSRDPIYSNKLGKFFEITPEK	SRDPIYSNKLGKLFEITPEKNPQLR	0.9446	0	P11827
	PFNLRSRDPIYSNKLGKFFEITPEK	SRNPIYSNNFGKFFEITPEKNPQLR	0.9304	0	F7J077

	PFNLRSRDPIYSNKLGKFFEITPEK	SRNPIYSNNFGKFFEITPEKNPQLR	0.9304	0	P25974
	PFNLRSRDPIYSNKLGKFFEITPEK	SRNPIYSNNFGKFFEITPEKNPQLR	0.9304	0	Q50JD8
9	PFNLRSRDPIYSNKLGKLFEITPEKNPQLR	SRDPIYSNKLGKLFEITPEKNPQLR	0.9829	0,05	P11827
	PFNLRSRDPIYSNKLGKLFEITPEKNPQLR	SRDPIYSNKLGKFFEITPEKNPQLR	0.9706	0,05	P0DO15
	PFNLRSRDPIYSNKLGKLFEITPEKNPQLR	SRDPIYSNKLGKFFEITPEKNPQLR	0.9706	0,05	P0DO16
	PFNLRSRDPIYSNKLGKLFEITPEKNPQLR	SRNPIYSNNFGKFFEITPEKNPQLR	0.9024	0,05	F7J077
	PFNLRSRDPIYSNKLGKLFEITPEKNPQLR	SRNPIYSNNFGKFFEITPEKNPQLR	0.9024	0,05	P25974
	PFNLRSRDPIYSNKLGKLFEITPEKNPQLR	SRNPIYSNNFGKFFEITPEKNPQLR	0.9024	0,05	Q50JD8
10	QSQVSELKYEGNWGPLVNPESQQGSPR	YEGNWGPLVNPESQQGSPRVKVA	0.9238	0,04	P02858
	QSQVSELKYEGNWGPLVNPESQQGSPR	SQSESYFVDAQPQQKEEGNKGRKGPLSSILR	0.9161	0,04	P11827
11	SQQLQNLRDYRILEFNSKPNTLLLPHHADADYLIVILNGTAILTLVNNDDR	ILEFNSKPNTLLLPNHADADYLIVILNGTAILSLVNNDDRDSYR	0.9673	0,07	P0DO15
	SQQLQNLRDYRILEFNSKPNTLLLPHHADADYLIVILNGTAILTLVNNDDR	ILEFNSKPNTLLLPNHADADYLIVILNGTAILSLVNNDDRDSYR	0.9673	0,07	P0DO16
12	TISSEDEPFNLRSRNPIYSNNFGKFFEITPEK	SRNPIYSNNFGKFFEITPEKNPQLR	0.9469	0,07	F7J077
	TISSEDEPFNLRSRNPIYSNNFGKFFEITPEK	SRNPIYSNNFGKFFEITPEKNPQLR	0.9469	0,07	F7J077
	TISSEDEPFNLRSRNPIYSNNFGKFFEITPEK	SRNPIYSNNFGKFFEITPEKNPQLR	0.9469	0,07	P25974
	TISSEDEPFNLRSRNPIYSNNFGKFFEITPEK	SRNPIYSNNFGKFFEITPEKNPQLR	0.9469	0,07	P25974
	TISSEDEPFNLRSRNPIYSNNFGKFFEITPEK	SRNPIYSNNFGKFFEITPEKNPQLR	0.9469	0,07	Q50JD8
	TISSEDEPFNLRSRNPIYSNNFGKFFEITPEK	SRNPIYSNNFGKFFEITPEKNPQLR	0.9469	0,07	Q50JD8
	TISSEDEPFNLRSRNPIYSNNFGKFFEITPEK	SRDPIYSNKLGKFFEITPEKNPQLR	0.9053	0,07	P0DO15
	TISSEDEPFNLRSRNPIYSNNFGKFFEITPEK	SRDPIYSNKLGKFFEITPEKNPQLR	0.9053	0,07	P0DO15
	TISSEDEPFNLRSRNPIYSNNFGKFFEITPEK	SRDPIYSNKLGKFFEITPEKNPQLR	0.9053	0,07	P0DO16
	TISSEDEPFNLRSRNPIYSNNFGKFFEITPEK	SRDPIYSNKLGKFFEITPEKNPQLR	0.9053	0,07	P0DO16
13	VLQRFNQRSPQLQNLRDYRILEFNSK	NQYGRIRVLQRFNQRSPQLQNLR	0.939	0,03	P0DO15
	VLQRFNQRSPQLQNLRDYRILEFNSK	NQYGRIRVLQRFNQRSPQLQNLR	0.939	0,03	P0DO16
	VLQRFNQRSPQLQNLRDYRILEFNSK	NQYGHVRVLQRFNKRSQQLQNLR	0.9222	0,03	P11827

Comparison between sequences found by mass spectrum and sequences found by *in silico* simulated digestion.

	In vitro sequences (LMMH)	In silico sequences (LMMH)	Cosine value	Sequence Difference	Protein
1	AGVQGENEEEDSGALVTVQ	NLQGENEEEDSGAIVTVK	0.9238	0,01	P04405
	NLQGENEEEDSGAIVTVK	KLQGENEEEEKGAIVTVK	0.9141	0	P11828
2	NLQGENEEEDSGAIVTVK	NLQGENEEEDSGAIVTVK	1	0	P04405
	NLQGENEEEDSGAIVTVK	NLQGENEGEDKGAIVTVK	0.9444	0	P04776

Supplementary Data S2 - Structure–activity relationships of soy-derived antioxidant and alpha-glucosidase inhibitor peptides identified by MS.

	Identified peptides by MS (<3kDa)	Activity†	PepRank
1	AASILSSHDPPVVLAKIDANEEKNKDLASQYDVR	alpha-glucosidase inhibitor	0.183
	AASILSSHDPPVVLAKIDANEEKN**KD**LASQYDVR	antioxidative	0.183
2	AGRISTLNSLTLPALRQFGLSAQY**VVLY**R	antioxidative	0.3256
	AGRISTLNSLTLPALRQFGLSAQYVV**LY**R	antioxidative	0.3256
3	AGRISTLNSLTLPALRQFQLSAQY**VVLY**K	antioxidative	0.2395
	AGRISTLNSLTLPALRQFQLSAQYVV**LY**K	antioxidative	0.2395
4	ANFLSSEDFEPPLIPSKTIPDPDDKKPEDWDER	alpha-glucosidase inhibitor	0.1372
	ANFLSSEDFEPPLIPSKTIPDPDDKKPEDWDER	alpha-glucosidase inhibitor	0.1372
	ANFLSSEDFEPPLIPSKTIPDPDDK**KP**EDWDER	antioxidative	0.1372
5	ARENPENPSIELGPEFKKVSNFLGRFK	alpha-glucosidase inhibitor	0.7585
	ARENPENPSI**EL**GPEFKKVSNFLGRFK	antioxidative	0.7585
	ARENPENPSIELGPE**FKK**VSNFLGRFK	antioxidative	0.7585
6	AVEAYLLAHPDYNMASGEQKFPPQQQQTQPGK	alpha-glucosidase inhibitor	0.1163
	AVEAYLLAHPDYNMASGEQKFPPQQQQTQPGK	alpha-glucosidase inhibitor	0.1163
	AVEAYLLAHPDYNMASGEQKFPPQQQQTQPGK	alpha-glucosidase inhibitor	0.1163
	AVEA**YLL**AHPDYNMASGEQKFPPQQQQTQPGK	antioxidative	0.1163
	AVE**AY**LLAHPDYNMASGEQKFPPQQQQTQPGK	antioxidative	0.1163
	AVEAYLL**AH**PDYNMASGEQKFPPQQQQTQPGK	antioxidative	0.1163
7	CPLTVVQSRN**EL**DKGIGTIISSPYRIR	antioxidative	0.4245
	CPLTVVQSRNELDKGIGTIISSPYR**IR**	antioxidative	0.4245
8	CTLNRNALRRPSYTNGPQE**IY**IQQGK	antioxidative	0.0795
9	DEKTLLFGDKPVTVFGHRNPEEIPWK	alpha-glucosidase inhibitor	0.1663
	DEKTLLFGD**KP**VTVFGHRNPEEIPWK	antioxidative	0.1663
	DEKTLLFGDKPVTVFGHRNPEEI**PW**K	antioxidative	0.1663

10	DGNVAPFVKSEPIPEANDEPVKVVVGNSLEDIVFK	alpha-glucosidase inhibitor	0.0251
	DGNVAPFVKSEPIPEANDEPVKVVVGNSLEDIVFK	alpha-glucosidase inhibitor	0.0251
	DGNVAPFVKSEPIPEANDEP**VKV**VVGNSLEDIVFK	antioxidative	0.0251
11	DLHEGGIKLPTEVISTIMPLPVVK**EL**FR	antioxidative	0.0073
	D**LH**EGGIKLPTEVISTIMPLPVVKELFR	antioxidative	0.0073
	D**LHE**GGIKLPTEVISTIMPLPVVKELFR	antioxidative	0.0073
	DLHEGG**IKL**PTEVISTIMPLPVVKELFR	antioxidative	0.0073
12	DPNSESRSNDVYLPRDEAFGHLKSSDFLTYGLK	alpha-glucosidase inhibitor	0.6349
	DPNSESRSNDVYLPRDEAFGH**LK**SSDFLTYG**LK**	antioxidative	0.6349
	DPNSESRSNDVYLPRDEAFGHLKSSDFL**TY**GLK	antioxidative	0.6349
	DPNSESRSND**VY**LPRDEAFGHLKSSDFLTYGLK	antioxidative	0.6349
	DPNSESRSNDVYLPRDEAFG**HL**KSSDFLTYGLK	antioxidative	0.6349
	DPNSESRSNDVYLPRDEAFGHLKS**SDF**LTYGLK	antioxidative	0.6349
13	DSYNLQSGDALRVPAGTT**YYV**VNPDNDENLR	antioxidative	0.3888
	DSYNLQSGDALRVPAGT**TY**YVVNPDNDENLR	antioxidative	0.3888
	DS**YNL**QSGDALRVPAGTTYYVVNPDNDENLR	antioxidative	0.3888
	DSYNLQSGDALRVPAG**TTYY**VVNPDNDENLR	antioxidative	0.3888
	DSYNLQSGDALRVPAGT**TYY**VVNPDNDENLR	antioxidative	0.3888
14	DSYRLQSGDALRVPSGTT**YYV**VNPDNNENLR	antioxidative	0.485
	DSYRLQSGDALRVPSGT**TY**YVVNPDNNENLR	antioxidative	0.485
	DSYRLQSGDALRVPSGTTYYVVNPDN**NEN**LR	antioxidative	0.485
	DSYR**LQSGDALRVPSGTTYY**VVNPDNNENLR	antioxidative	0.485
	DSYRLQSGDALRVPSG**TTYY**VVNPDNNENLR	antioxidative	0.485
	DSYRLQSGDALRVPSGT**TYY**VVNPDNNENLR	antioxidative	0.485
15	DWVFTDQALPADLIKRGMAIEDPSCPHGIR	alpha-glucosidase inhibitor	0.3346
	DWVFTDQALPADLIKRGMAIEDPSCPHG**IR**	antioxidative	0.3346
	DWVFTDQALPADLIKRGMAIEDPSC**PHG**IR	antioxidative	0.3346

16	ELAFNYPSEMVNGVSERKESLFFPFELPSEER	alpha-glucosidase inhibitor	0.0578
	ELAFNYPSEMVNGVSERKESLFFPF**EL**PSEER	antioxidative	0.0578
17	ELAFNYPSEMVNGVSERKESLFFPFELPSEERGR	alpha-glucosidase inhibitor	0.1008
	ELAFNYPSEMVNGVSERKESLFFPF**EL**PSEERGR	antioxidative	0.1008
18	EMIAGVNPCVIRGLEEFPPKSNLDPAIYGDQSSK	alpha-glucosidase inhibitor	0.1578
	EMIAGVNPCV**IR**GLEEFPPKSNLDPAIYGDQSSK	antioxidative	0.1578
	EMIAGVNPCVIRGLEEFPPKSNLDPA**IY**GDQSSK	antioxidative	0.1578
19	EMVAGVNPCVIRGLQEFPPKSNLDPTIYGEQTSK	alpha-glucosidase inhibitor	0.1908
	EMVAGVNPCV**IR**GLQEFPPKSNLDPTIYGEQTSK	antioxidative	0.1908
	EMVAGVNPCVIRGLQEFPPKSNLDPT**IY**GEQTSK	antioxidative	0.1908
20	FFEITPEKNPQLRDLDIFLSSVDINEGALLLPHFNSK	alpha-glucosidase inhibitor	0.0259
	FFEITPEKNPQLRDLDIFLSSVDINEGAL**LLPH**FNSK	antioxidative	0.0259
	FFEITPEKNPQLRDLDIFLSSVDINEGALLL**PHF**NSK	antioxidative	0.0259
21	FPLLVLLGTVFLASVCVS**LK**VREDENNPFYFR	antioxidative	0.9836
22	FQTLFKNQHG**HL**RVLQRFDQRSPQLENLR	antioxidative	0.5471
23	GGGIEVDSTGKEICPLTVVQSPN**EL**DKGIGLVFTSPLHALFIAER	antioxidative	0.0836
	GGGIEVDSTGKEICPLTVVQSPNELDKGIGLVFTSP**LH**ALFIAER	antioxidative	0.0836
	GGGIEVDSTGKEICPLTVVQSPNELDKGIGLVFTSP**LHA**LFIAER	antioxidative	0.0836
24	GKGRVRVVNMASGGKKFPPQQQQTQPGK	alpha-glucosidase inhibitor	0.2613
25	HASDEVYLGERDNPNWTSDTRALEAFK	alpha-glucosidase inhibitor	0.1246
	HASDE**VY**LGERDNPNWTSDTRALEAFK	antioxidative	0.1246
	HASDEV**YLG**ERDNPNWTSDTRALEAFK	antioxidative	0.1246
26	ILARSLGVSNRTIDELLRSPEDSIIFR	alpha-glucosidase inhibitor	0.0514
	ILARSLGVSNRTID**EL**LRSPEDSIIFR	antioxidative	0.0514
	ILARSLGVSNRTIDE**LLR**SPEDSIIFR	antioxidative	0.0514
27	KFPPQQQQTQPGKEHAMNPVPQFASPDYK	alpha-glucosidase inhibitor	0.1589
	KFPPQQQQTQPGKEHAMNPVPQFASP**DYK**	antioxidative	0.1589

28	KLDE**YLL**PRSYITGEIVQQLIYGGLYWYQATK	antioxidative	0.0698
	KLDEYLLPRSYITGEIVQQLIYGG**LY**WYQATK	antioxidative	0.0698
	KLDEYLLPRSYITGEIVQQL**IY**GGLYWYQATK	antioxidative	0.0698
	KLDEYLLPRSYITGEIVQQLIYGGLY**WY**QATK	antioxidative	0.0698
	KLDEYLLPRSYITGEIVQQLIYGGL**YWY**QATK	antioxidative	0.0698
29	KPSQPQEAEERPSEGIGETVRQYAQKPK	alpha-glucosidase inhibitor	0.7696
	KPSQPQEAEERPSEGIGETVRQYAQ**KP**K	antioxidative	0.7696
30	LIGRLYHLPHKDRKESFFFPF**EL**PR	antioxidative	0.0837
	LIGRLY**HLPH**KDRKESFFFPFELPR	antioxidative	0.0837
	LIGRLY**HL**PHKDRKESFFFPFELPR	antioxidative	0.0837
	LIGR**LY**HLPHKDRKESFFFPFELPR	antioxidative	0.0837
	LIGRLYHL**PHK**DRKESFFFPFELPR	antioxidative	0.0837
	LIGRLYHLPH**KD**RKESFFFPFELPR	antioxidative	0.0837
31	LMAVTLYDLSSASG**LK**KLDEYLLPRSYITGYQATK	antioxidative	0.1903
	LMAVTLYDLSSASGLKKLDE**YLL**PRSYITGYQATK	antioxidative	0.1903
	LMAVT**LY**DLSSASGLKKLDEYLLPRSYITGYQATK	antioxidative	0.1903
32	LNA**LKP**DNRIESEGGFIETWNPNNKPFQCAGVALSR	antioxidative	0.1392
	LNA**LK**PDNRIESEGGFIETWNPNNKPFQCAGVALSR	antioxidative	0.1392
	LNAL**KP**DNRIESEGGFIETWNPNN**KP**FQCAGVALSR	antioxidative	0.1392
	LNALKPDNRIESEGGFIE**TW**NPNNKPFQCAGVALSR	antioxidative	0.1392
33	LPTEVISTIMPLPVVK**EL**FRTDGEQVLK	antioxidative	0.0154
	LPTEVISTIMPLPVVKELFRTDGEQV**LK**	antioxidative	0.0154
34	LSAEFGSLRKNAMFVPH**YNL**NANSIIYALNGR	antioxidative	0.2263
	LSAEFGSLRKNAMFVPHYNLNANSI**IY**ALNGR	antioxidative	0.2263
	LSAEFGSLRKNAMFV**PHY**NLNANSIIYALNGR	antioxidative	0.2263
	LSAEFGSLRKNAM**FVPH**YNLNANSIIYALNGR	antioxidative	0.2263
35	LSAQYGSLRKNAMFVPHYTLNANSI**IY**ALNGR	antioxidative	0.2808

	LSAQYGSLRKNAMFV**PHY**TLNANSIIYALNGR	antioxidative	0.2808
	LSAQYGSLRKNAM**FVPH**YTLNANSIIYALNGR	antioxidative	0.2808
36	LTGMAFRVPTVDVSVVDLTVRLEKEASYDEIK	alpha-glucosidase inhibitor	0.0792
37	N**EL**DKGIGTIISSPYRIRFIAEGHPLSLK	antioxidative	0.0188
	NELDKGIGTIISSPYR**IR**FIAEGHPLSLK	antioxidative	0.0188
	NELDKGIGTIISSPYRIRFIAEGHPLS**LK**	antioxidative	0.0188
38	NKARENPENPSIELGPEFKKVSNFLGR	alpha-glucosidase inhibitor	0.4145
	NKARENPENPSI**EL**GPEFKKVSNFLGR	antioxidative	0.4145
	NKARENPENPSIELGPE**FKK**VSNFLGR	antioxidative	0.4145
39	NPEEIPWKSTGADIIVESTGVFTDKDKAAAHLK	alpha-glucosidase inhibitor	0.0076
	NPEEIPWKSTGADIIVESTGVFTDKDKAAAHLK	alpha-glucosidase inhibitor	0.0076
	NPEEIPWKSTGADIIVESTGVFTDKDKAAAHLK	alpha-glucosidase inhibitor	0.0076
	NPEEIPWKSTGADIIVESTGVFTDKDKAAAH**LK**	antioxidative	0.0076
	NPEEIPWKSTGADIIVESTGVFTDKDKAAA**HL**K	antioxidative	0.0076
	NPEEIPWKSTGADIIVESTGVFTDKDKAA**AH**LK	antioxidative	0.0076
	NPEEIPWKSTGADIIVESTGVFTD**KD**KAAAHLK	antioxidative	0.0076
	NPEEI**PW**KSTGADIIVESTGVFTDKDKAAAHLK	antioxidative	0.0076
40	NPFLFGSNRFETLFKNQYGR**IR**VLQR	antioxidative	0.3902
41	PDNELQILIDGEEKKKANFLSSEDFEPPLIPSK	alpha-glucosidase inhibitor	0.0313
	PDN**EL**QILIDGEEKKKANFLSSEDFEPPLIPSK	antioxidative	0.0313
42	PFNLGSRDP**IY**SNKLGKLFEITQRNPQLR	antioxidative	0.2904
43	PFNLRSRDPIYSNKLGKFFEITPEK	alpha-glucosidase inhibitor	0.0894
	PFNLRSRDP**IY**SNKLGKFFEITPEK	antioxidative	0.0894
44	PFNLRSRDPIYSNKLGKLFEITPEKNPQLR	alpha-glucosidase inhibitor	0.0699
	PFNLRSRDP**IY**SNKLGKLFEITPEKNPQLR	antioxidative	0.0699
45	PLRLPLQDVYKIGGIGTVPVGRVETGVLK	alpha-glucosidase inhibitor	0.1322
	PLRLPLQDVYKIGGIGTVPVGRVETGV**LK**	antioxidative	0.1322

	PLRLPLQD**VY**KIGGIGTVPVGRVETGVLK	antioxidative	0.1322
	PLR**LPL**QDVYKIGGIGTVPVGRVETGVLK	antioxidative	0.1322
46	PQHPEREPQQPGEKEEDEDEQPRPIPFPR	alpha-glucosidase inhibitor	0.8585
	PSDKPLRLPLQDVYKIGGIGTVPVGRVETGVLK	alpha-glucosidase inhibitor	0.2355
	PSDKPLRLPLQDVYKIGGIGTVPVGRVETGV**LK**	antioxidative	0.2355
47	PSD**KP**LRLPLQDVYKIGGIGTVPVGRVETGVLK	antioxidative	0.2355
	PSDKPLRLPLQD**VY**KIGGIGTVPVGRVETGVLK	antioxidative	0.2355
	PSDKPLR**LPL**QDVYKIGGIGTVPVGRVETGVLK	antioxidative	0.2355
	QIR**EL**SKHAKSSSRKTISSEDKPFNLR	antioxidative	0.2057
48	Q**IR**ELSKHAKSSSRKTISSEDKPFNLR	antioxidative	0.2057
	QIRELSKHAKSSSRKTISSED**KP**FNLR	antioxidative	0.2057
	QSQVSELKYEGNWGPLVNPESQQGSPR	alpha-glucosidase inhibitor	0.441
	QSQVS**EL**KYEGNWGPLVNPESQQGSPR	antioxidative	0.441
49	QSQVSE**LK**YEGNWGPLVNPESQQGSPR	antioxidative	0.441
	QSQVSELKYEGN**WG**PLVNPESQQGSPR	antioxidative	0.441
	QSQVSELKYEGNWGPL**VNPESQQGSPR**	antioxidative	0.441
	SAWMTDEEFAREMVAGVNPCVIRGLQEFPPK	alpha-glucosidase inhibitor	0.4581
50	SAWMTDEEFAREMVAGVNPCV**IR**GLQEFPPK	antioxidative	0.4581
	S**AW**MTDEEFAREMVAGVNPCVIRGLQEFPPK	antioxidative	0.4581
	SEPIPEANDEPVKVVVGNSLEDIVFKSGK	alpha-glucosidase inhibitor	0.0937
51	SEPIPEANDEPVKVVVGNSLEDIVFKSGK	alpha-glucosidase inhibitor	0.0937
	SEPIPEANDEP**VKV**VVGNSLEDIVFKSGK	antioxidative	0.0937
52	SPEDSIIFRCSSCAEEEEKIMKEEEEEEESK	alpha-glucosidase inhibitor	0.085
	SPEEGVEWEQEEARTASREGEKKTPR	alpha-glucosidase inhibitor	0.6862
53	SPEEGVEWEQEEARTASREGEKKTPR	alpha-glucosidase inhibitor	0.6862
	SPEEGVEWEQEEARTASREGEKKTPR	alpha-glucosidase inhibitor	0.6862
54	SQQLQNLRDYRILEFNSKPNTLLLPHHADADYLIVILNGTAILTLVNNDDR	alpha-glucosidase inhibitor	0.0285

	SQQLQNLRDYRILEFNS**KP**NTLLLPHHADADYLIVILNGTAILTLVNNDDR	antioxidative	0.0285
	SQQLQNL**RDY**RILEFNSKPNTLLLPHHADADYLIVILNGTAILTLVNNDDR	antioxidative	0.0285
	SQQLQNLRDYRILEFNSKPNTLLL**PHH**ADADYLIVILNGTAILTLVNNDDR	antioxidative	0.0285
	SQQLQNLRDYRILEFNSKPNTL**LLPH**HADADYLIVILNGTAILTLVNNDDR	antioxidative	0.0285
	SQQLQNLRDYRILEFNSKPNTLL**LPHH**ADADYLIVILNGTAILTLVNNDDR	antioxidative	0.0285
	SQQLQNLRDYRILEFNSKPNTLLLP**HH**ADADYLIVILNGTAILTLVNNDDR	antioxidative	0.0285
	SQQLQNLRDYRILEFNSKPNTL**LLPHH**ADADYLIVILNGTAILTLVNNDDR	antioxidative	0.0285
	SQQLQNLRDYRILEFNSKPNTL**LLPHHADADY**LIVILNGTAILTLVNNDDR	antioxidative	0.0285
55	SSSRKTISSED**KP**FNLRSRDPIYSNK	antioxidative	0.3582
	SSSRKTISSEDKPFNLRSRDP**IY**SNK	antioxidative	0.3582
56	SSWWPFGGES**KP**QFNIFSKRPTISNGYGR	antioxidative	0.7771
	SSWWPF**GGE**SKPQFNIFSKRPTISNGYGR	antioxidative	0.7771
57	TISSEDEPFNLRSRNPIYSNNFGKFFEITPEK	alpha-glucosidase inhibitor	0.2268
	TISSEDEPFNLRSRNP**IY**SNNFGKFFEITPEK	antioxidative	0.2268
58	TNDTPMIGTLAGANSLLNALPEEVIQHTFNLKSQQARQIKNNNPFKFLVPPQESQK	alpha-glucosidase inhibitor	0.1879
	TNDTPMIGTLAGANSLLNALPEEVIQHTFNLKSQQARQIKNNNPFKFLVPPQESQK	alpha-glucosidase inhibitor	0.1879
	TNDTPMIGTLAGANSLLNALPEEVIQHTFN**LK**SQQARQIKNNNPFKFLVPPQESQK	antioxidative	0.1879
59	TSLPEWVRIGFSAATGLDIPGESHDVLSWSFASNLPHASSNIDPLDLTSFVLHEAI	alpha-glucosidase inhibitor	0.0321
	TSLPEWVRIGFSAATGLDIPGESHDVLSWSFASNLPHASSNIDPLDLTSFVLHEAI	alpha-glucosidase inhibitor	0.0321
	TSLPEWVRIGFSAATGLDIPGESHDVLSWSFASNLPHASSNIDPLDLTSFV**LH**EAI	antioxidative	0.0321
	TSLPEWVRIGFSAATGLDIPGESHDVLSWSFASNLPHASSNIDPLDLTSFV**LHE**AI	antioxidative	0.0321
	TSLPEWVRIGFSAATGLDIPGESHDVLSWSFASNL**PHA**SSNIDPLDLTSFVLHEAI	antioxidative	0.0321
60	VLQRFNQRSPQLQNL**RDY**RILEFNSK	antioxidative	0.2854
61	WQEQEDEDEDEDEEYEQTPSYPPR	alpha-glucosidase inhibitor	0.8585
	WQEQEDEDEDEDEEYEQTPSYPPR	alpha-glucosidase inhibitor	0.8585
62	YQNSNIEIHTFNQSQYPRLVVEDFLPLPSK	alpha-glucosidase inhibitor	0.0066
	YQNSNIEIHTFNQSQYPRLVVEDFLPLPSK	alpha-glucosidase inhibitor	0.0066

	YQNSNIEIHTFNQS**QYP**RLVVEDFLPLPSK	antioxidative	0.0066
	YQNSNIEIHTFNQSQYPRLVVEDF**LPL**PSK	antioxidative	0.0066
63	YSVEMSAVVYKDWVFTDQALPADLIKR	alpha-glucosidase inhibitor	0.0808
	YSVEMSAVVYKDWVFTDQALPADLIKR	alpha-glucosidase inhibitor	0.0808
	YSVEMSAV**VY**KDWVFTDQALPADLIKR	antioxidative	0.0808
	YSVEMSAVVY**KD**WVFTDQALPADLIKR	antioxidative	0.0808
64	CSSCAEEEEKIMKEEEEEEESKKEEEER	***	***
65	EDENNPFYFRSSNSFQTLFENQNVR	***	***
66	EDENNPFYLRSSNSFQTLFENQNGR	***	***
67	EPQQPGEKEEDEDEQPRPIPFPRPR	***	***
68	TPGPGAQSALRALARSGMKIGRIEDVTPIPSDSTR	***	***
69	YVDENFEGTPLFPRDPAKKEFGEQLISHVDTFSR	***	***

*Sequences in bold represent antioxidant activity and sequences in red represent alpha glucosidase inhibitory activity.
*** No activity.
†Biological activity identified by the BIOPEP-UWM database (Minkiewicz et al., 2019).

	Identified peptides by MS (<3kDa)	Activity†	PepRank
1	AIVVLVINEGEANIELVGIKEQQQR	alpha-glucosidase inhibitor	0.0555
	AIVVLVINEGEANI**EL**VGIKEQQQR	antioxidative	0.0555
2	ALVVLVLNEWANL**EL**VGLKEQQQR	antioxidative	0.4653
	ALVVLVLNEWANLELVG**LK**EQQQR	antioxidative	0.4653
3	AVGSATGM**EL**VTNVALSEVSLK	antioxidative	0.0382
	AVGSATGMELVTNVALSEVS**LK**	antioxidative	0.0382
4	QAAGQAGNAATGAG**AY**	antioxidative	0.314
5	S**IR**	antioxidative	0.2922
6	VVPPA	alpha-glucosidase inhibitor	0.2322
7	AGVQGENEEEDSGALVTVQ	***	***
8	EDASLAQVNGGQ	***	***
9	FQTLIDLSVIEILSR	***	***
10	NLQGENEEEDSGAIVTVK	***	***
11	QKNVSDTPYVT	***	***
12	CCK	***	***
13	CCQ	***	***
14	EF	***	***
15	HR	***	***
16	ISK	***	***
17	ISQ	***	***
18	IT	***	***
19	LSK	***	***
20	LSQ	***	***
21	LT	***	***
22	ME	***	***

23	NET	***	***
24	NGT	***	***
25	NPA	***	***
26	NVH	***	***
27	SLR	***	***
28	SPK	***	***
29	TI	***	***
30	TL	***	***
31	VTK	***	***
32	VTQ	***	***

*Sequences in bold represent antioxidant activity and sequences in red represent alpha glucosidase inhibitory activity.
*** No activity.
†Biological activity identified by the BIOPEP-UWM database (Minkiewicz et al., 2019).

SUPPLEMEMTARY MATERIAL

CAPÍTULO IV

ANTIPROLIFERATIVE EFFECT OF PEPTIDE FRACTIONS FROM BRAZILIAN SOY PROTEIN HYDROLYSATES IN PROSTATE CANCER CELLS

Supplementary Table 1 – Peptides of soybean hydrolysate identified by Mass Spectometry. Bold and red sequences represented peptides that have anticancer activity by BIOPEP-UWM.

HMMH Fraction	LMMH Fraction
AASILSSHDPPVVLAKIDANEEKNKDLASQYDVR	AGVQGENEEEDSGALVTVQ
AGRISTLNSLTLPALRQFGLSAQYVVLYR	AIVVLVINEGEANIELVGIKEQQQR
AGRISTLNSLTLPALRQFQLSAQYVVLYK	ALVVLVLNEWANLELVGLKEQQQR
ANFLSSEDFEPPLIPSKTIPDPDDKKPEDWDER	AVGSATGMELVTNVALSEVSLK
ARENPENPSIELGPEFKKVSNFLGRFK	CCK
AVEAYLLAHPDYNMASGEQKFPPQQQQTQPGK	CCQ
CPLTVVQSRNELDKGIGTIISSPYRIR	EDASLAQVNGGQ
CSSCAEEEEKIMKEEEEEEESKKEEEER	EF
CTLNRNALRRPSYTNGPQEIYIQQGK	FQTLIDLSVIEILSR
DEKTLLFGDKPVTVFGHRNPEEIPWK	HR
DGNVAPFVKSEPIPEANDEPVKVVVGNSLEDIVFK	ISK
DLHEGGIKLPTEVISTIMPLPVVKELFR	ISQ
DPNSESRSNDVYLPRDEAFGHLKSSDFLTYGLK	IT
DSYNLQSGDALRVPAGTTYYVVNPDNDENLR	LSK
DSYRLQSGDALRVPSGTTYYVVNPDNNENLR	LSQ
DWVFTDQALPADLIKRGMAIEDPSCPHGIR	LT
EDENNPFYFRSSNSFQTLFENQNVR	ME
EDENNPFYLRSSNSFQTLFENQNGR	NET
ELAFNYPSEMVNGVSERKESLFFPFELPSEER	NGT
ELAFNYPSEMVNGVSERKESLFFPFELPSEERGR	NLQGENEEEDSGAIVTVK
EMIAGVNPCVIRGLEEFPPKSNLDPAIYGDQSSK	NPA
EMVAGVNPCVIRGLQEFPPKSNLDPTIYGEQTSK	NVH
EPQQPGEKEEDEDEQPRPIPFPRPR	QAAGQAGNAATGAGAY

FFEITPEKNPQLRDLDIFLSSVDINEGALLLPHFNSK
FPLLVLLGTVFLASVCVSLKVREDENNPFYFR
FQTLFKNQHGHLRVLQRFDQRSPQLENLR
GGGIEVDSTGKEICPLTVVQSPNELDKGIGLVFTSPLHALFIAER
GKGRVRVVNMASGGKKFPPQQQQTQPGK
HASDEVYLGERDNPNWTSDTRALEAFK
ILARSLGVSNRTIDELLRSPEDSIIFR
KFPPQQQQTQPGKEHAMNPVPQFASPDYK
KLDEYLLPRSYITGEIVQQLIYGGLYWYQATK
KPSQPQEAEERPSEGIGETVRQYAQKPK
LIGRLYHLPHKDRKESFFFPFELPR
LMAVTLYDLSSASGLKKLDEYLLPRSYITGYQATK
LNALKPDNRIESEGGFIETWNPNNKPFQCAGVALSR
LPTEVISTIMPLPVVKELFRTDGEQVLK
LSAEFGSLRKNAMFVPHYNLNANSIIYALNGR
LSAQYGSLRKNAMFVPHYTLNANSIIYALNGR
LTGMAFRVPTVDVSVVDLTVRLEKEASYDEIK
NELDKGIGTIISSPYRIRFIAEGHPLSLK
NKARENPENPSIELGPEFKKVSNFLGR
NPEEIPWKSTGADIIVESTGVFTDKDKAAAHLK
NPFLFGSNRFETLFKNQYGRIRVLQR
PDNELQILIDGEEKKKANFLSSEDFEPPLIPSK
PFNLGSRDPIYSNKLGKLFEITQRNPQLR
PFNLRSRDPIYSNKLGKFFEITPEK
PFNLRSRDPIYSNKLGKLFEITPEKNPQLR
PLRLPLQDVYKIGGIGTVPVGRVETGVLK
PQHPEREPQQPGEKEEDEDEQPRPIPFPR
PSDKPLRLPLQDVYKIGGIGTVPVGRVETGVLK
QKNVSDTPYVT
SIR
SLR
SPK
TI
TL
VTK
VTQ
VVPPA

QIRELSKHAKSSSRKTISSEDKPFNLR

QSQVSELKYEGNWGPLVNPESQQGSPR

SAWMTDEEFAREMVAGVNPCVIRGLQEFPPK

SEPIPEANDEPVKVVVGNSLEDIVFKSGK

SPEDSIIFRCSSCAEEEEKIMKEEEEEEESK

SPEEGVEWEQEEARTASREGEKKTPR

SQQLQNLRDYRILEFNSKPNTLLLPHHADADYLIVILNGTAILTLVNNDDR

SSSRKTISSEDKPFNLRSRDPIYSNK

SSWWPFGGESKPQFNIFSKRPTISNGYGR

TISSEDEPFNLRSRNPIYSNNFGKFFEITPEK

TNDTPMIGTLAGANSLLNALPEEVIQHTFNLKSQQARQIKNNNPFKFLVPPQESQK

TPGPGAQSALRALARSGMKIGRIEDVTPIPSDSTR

TSLPEWVRIGFSAATGLDIPGESHDVLSWSFASNLPHASSNIDPLDLTSFVLHEAI

VLQRFNQRSPQLQNLRDYRILEFNSK

WQEQEDEDEDEDEEYEQTPSYPPR

YQNSNIEIHTFNQSQYPRLVVEDFLPLPSK

YSVEMSAVVYKDWVFTDQALPADLIKR

YVDENFEGTPLFPRDPAKKEFGEQLISHVDTFSR