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THALYNE MARIANE SANTANA DE FARIA

BIODISPONIBILIDADE DE PEPTÍDEOS DE ISOLADOS DO SORO DE LEITE E DA SEMENTE DE GIRASSOL APÓS DIGESTÃO *IN VITRO* EM CULTURA DE LINHAGEM CACO-2

CAMPINAS
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RESUMO

As fontes de proteína animal e vegetal podem produzir um conjunto variado de peptídeos bioativos no sistema digestivo com maior ou menor capacidade de absorção. Para exibir as suas funcionalidades, os peptídeos alimentares devem sobreviver ao trânsito gastrointestinal tornando essenciais estudos *in vitro* para avaliar a resistência à degradação gastrointestinal. Este estudo comparou a bioacessibilidade, biodisponibilidade e bioatividade de digeridos peptídicos provenientes do isolado do soro de leite (DSL) e da semente do girassol (DG). Após hidrólise por digestão simulada os peptídeos foram caracterizados quanto ao grau de hidrólise e tamanho molecular por cromatografia de exclusão de tamanho (FPLC). Os hidrolisados foram testados quanto a sua toxicidade em diferentes concentrações (0,01 a 2 mg/mL) pelo ensaio de MTT. As células Caco-2 foram cultivadas em placas transwell durante 19 dias e a monocamada foi avaliada por TEER até chegarem a valores >250 Ω/cm^2. O meio apical e basolateral foi coletado e realizada quantificação por aminoácidos totais, bem como identificação dos peptídeos por cromatografia líquida de alta eficiência (HPLC-MS/MS). Uma vez sequenciados, os peptídeos foram analisados em relação a suas características físico-químicas e a bioatividade por técnicas *in sílico*. Utilizando o Infogest 2.0 obtivemos a formação de digeridos com alta solubilidade proteica (>60%) uma vez que, o DSL apresentou grau de hidrólise de 66,01% enquanto o DG apresentou 61,72%. A análise de tamanho molecular revelou que o DSL teve predominância de peptídeos menores, entre 0,2 a 0,4 kDa, enquanto que o DG teve peptídeos com uma maior distribuição entre 1,0 a 0,4 kDa. O estudo de biodisponibilidade seguiu-se com as concentrações 0,01 e 1 mg/mL uma vez que, nenhuma concentração dos digeridos comprometeu a viabilidade celular. Os peptídeos do soro de leite foram ricos em leucina, valina, glutamato e aspartato, enquanto os peptídeos de girassol apresentaram leucina, treonina, aspartato e glutamato como aminoácidos predominantes. Em relação a análise de transportabilidade de peptídeos, cerca de 70% dos peptídeos do DSL foram transportados, enquanto que o DG apresentou ‘menor taxa de absorção. No entanto, maiores concentrações de hidrolisados de DG induziram maior biodisponibilidade de peptídeos. As características físico-químicas dos peptídeos foram diferentes entre os compartimentos. Os peptídeos biodisponíveis em ambas as amostras foram

positivamente hidrofóbicos com tamanho molecular intermediário, sugerindo um mecanismo de transporte independente de carreador. A análise *in sílico* da bioatividade mostrou que 60% dos peptídeos biodisponíveis têm maior previsibilidade para atuarem como composto anti-inflamatório. Dois peptídeos (TPEVDDEALEK e YLGYLEQLLR) da amostra de soro de leite cruzaram para a câmara basolateral da monocamada Caco-2. Sendo o primeiro identificado com atividade antioxidante, inibitória da dipeptidil peptidase-4 (DPP-4) e antimicrobiana. O segundo tem efeito ansiolítico. Nossos resultados mostram que a digestão de proteínas vegetais e animais originam diferentes padrões de peptídeos, com diferentes taxas de biodisponibilidade. No entanto, a concentração e o processamento das amostras vegetais podem ser decisivos para melhor absorção. Este estudo é importante para avaliar o impacto da variação alimentar em novos produtos proteicos alimentares.

Palavras-chave: Digerido do soro do leite; Digerido do girassol; Peptídeos bioativos; Células Caco-2; Transporte transepitelial.

ABSTRACT

Animal and plant protein sources can produce a diverse set of bioactive peptides in the digestive system with greater or lesser absorption capacity. To exhibit their functionalities, dietary peptides must survive gastrointestinal transit, making in vitro studies essential to evaluate their resistance to gastrointestinal degradation. This study evaluated the bioaccessibility and bioavailability of peptide digests from whey isolate (DSL) and sunflower (DG) in Caco-2 cell culture. After hydrolysis by simulated digestion, the peptides were characterized for the degree of hydrolysis and molecular size by size exclusion chromatography (FPLC). The hydrolysates were tested for their toxicity at different concentrations (0.01 to 2 mg/mL) by the MTT assay. Caco-2 cells were cultured in transwell plates for 19 days and the monolayer was evaluated by TEER until reaching values >250 Ω. The apical and basolateral medium was collected and quantified for total amino acids, as well as peptide identification by high-performance liquid chromatography (HPLC-MS/MS). Once sequenced, the peptides were analyzed for their physicochemical characteristics and bioactivity by in silico techniques. Using Infogest 2.0, we obtained the formation of digests with high protein solubility (>60%), since DSL presented a degree of hydrolysis of 66.01%, while DG presented 61.72%. Molecular size analysis revealed that DSL had a predominance of smaller peptides, between 0.2 and 0.4 kDa, while DG had peptides with a greater distribution between 0.2 and kDa. The bioavailability study followed with concentrations of 0.01 and 1 mg/mL, since no concentration of the digests compromised cell viability. The proportion of total amino acid content was different between the apical and basolateral compartments. Some amino acids with hydrophobic characteristics or positively charged showed greater transportability between the chambers. Regarding the peptide transportability analysis, DSL showed a higher absorption rate than DG. However, higher concentrations of DG hydrolysates induced greater peptide bioavailability. The amino acid sequence of the bioactive peptide showed different profiles. The whey peptides were rich in leucine, valine, glutamate and aspartate, while the sunflower peptides presented leucine and threonine as predominant amino acids. The physicochemical characteristics of the peptides were different between the compartments. The bioavailable peptides in both samples were positively hydrophobic with intermediate molecular size, suggesting a carrier-independent transport mechanism.

In silico analysis of bioactivity showed that the bioavailable peptides have greater predictability to act as anti-inflammatory compounds. Our results show that the digestion of plant and animal proteins produces different peptide patterns, with different bioavailability rates. However, the concentration and processing of plant samples can be decisive for better absorption. This study is important to evaluate the impact of dietary variation on new protein food products.

Keywords: Whey Protein digestion; Sunflower digestion; Bioactive peptides; Caco-2 cells; Transepithelial transport.

SUMÁRIO

1 INTRODUÇÃO

A descoberta de novas propriedades oriundas do isolamento e hidrólise das proteínas animais e vegetais, são de grande interesse na indústria de alimentos funcionais devido aos seus benefícios à saúde. No contexto de pesquisas envolvendo proteínas de origem animal tem se visto que, os produtos lácteos têm se tornado o principal alvo de estudos por serem produtos de alto valor nutricional, possuindo também uma fonte de peptídeos com atividades biológicas (Dias et al., 2019). Dentre esses, pode-se destacar o soro do leite bovino que, atualmente tem sido amplamente utilizado como suplemento em produtos alimentícios devido a sua elevada carga nutricional (Corrochano et al., 2018).

Já tratando-se da proteína de origem vegetal, existem poucos dados documentados acerca de sua biodisponibilidade, isto pode ocorrer devido ao fato de que os procedimentos para isolamento de peptídeos de matrizes vegetais, são mais complexos do que os empregados para outras matrizes biológicas. Quimicamente, a biomassa vegetal é composta por muitas substâncias tais como, pigmentos, açúcares, gorduras, taninos etc. o que dificulta o processo de isolamento dos peptídeos. Entretanto, é sabido que produtos de origem natural desempenham uma gama de benefícios para o organismo por apresentarem atividades antioxidantes, antibacteriana, anti-inflamatória e entre outras (Picchi et al., 2009).

Nesse sentido, destaca-se sobre a importância do girassol que é conhecido no setor de alimentos devido ao seu emprego na produção de óleos e produtos de panificação. A torta proveniente do produto desengordurado é rica em proteínas e apresenta em sua constituição compostos bioativos que podem trazer benefícios a saúde do consumidor (Lai et al., 2017). Nota-se então, a necessidade de se avaliar a biodisponibilidade de um determinado nutriente, seja ele de origem vegetal ou animal.

Ao considerar os benefícios fisiológicos das proteínas do soro de leite e do girassol é importante ressaltar que, estas não atingem o intestino em sua forma integra. A digestão das proteínas no sistema gastrointestinal formará peptídeos de tamanho pequeno e aminoácidos livres os quais serão absorvidos pela barreira gastrointestinal até chegar à corrente sanguínea e, somente assim

poderão exercer atividades bioativas. Porém, é limitado o conhecimento sobre até que ponto os peptídeos derivados da degradação de proteínas vegetais e animais são absorvidos pelo trato gastrointestinal e pouco se tem na literatura sobre o assunto. Ressalta-se então, a importância de modelos de linhagem celulares, especificamente, as células Caco-2 cuja linhagem foi originalmente obtida de um adenocarcinoma colorretal epitelial de um homem adulto. Em condições de cultivo, essas células sofrem diferenciação espontânea, o que possibilita a formação de uma monocamada, apresentado características de enterócitos maduros, isto é, características essenciais para a absorção e digestão dos nutrientes (Liang et al., 2022).

Pesquisas envolvendo o estudo de transporte de nutrientes, constantemente empregam a linhagem celular Caco-2 por serem culturas celulares que apresentam similaridade com às células epiteliais do intestino delgado, tanto morfologicamente quanto bioquimicamente (Liang et al., 2022). Além disso, o modelo é a alternativa mais viável para a substituição de animais em experimentos laboratoriais pois, os métodos *in vitro* são mais baratos, mais rápidos e mais simples. É importante ressaltar também, que por meio dos métodos *in vitro* é possível ter o controle das condições experimentais, evitando assim a variabilidade existentes entre o metabolismo animal e humano em estudos *in vivo* (Hur et al., 2011).

Nesse sentido, compreender o comportamento de absorção de peptídeos através da barreira intestinal se faz necessário para validar as potenciais funções fisiológicas, bem como o mecanismo de internalização dos peptídeos.

Com base nas premissas apresentadas, o presente estudo comparou quais peptídeos derivados da digestão *in vitro* da proteína animal (isolado do soro do leite) e vegetal (girassol), são capazes de resistir ao processo de digestão e atravessar monocamadas de células Caco-2 intestinais humanas e por fim estimar a biodisponibilidade dos peptídeos translocados em relação ao seu potencial bioativo por análises *in sílico.*

2 OBJETIVOS

2.1 Objetivo principal

Comparar a bioacessibilidade, biodisponibilidade e bioatividade de peptídeos derivados da proteína do soro do leite e semente do girassol por meio de análises de digestão simulada, modelo de barreira gastrointestinal e bioatividade *in sílico*.

2.2 Objetivos específicos

- Avaliar o metabolismo de proteínas alternativas de origem vegetal em comparação com proteína animal em relação a sua capacidade de serem digeridos, absorvidos e formarem peptídeos bioativos
- Estimar o impacto da aplicação de proteínas alternativas vegetais em produtos proteicos em relação a sua capacidade nutricional e bioativa.
- Obtenção de isolados proteicos da farinha desengordurada do girassol com redução de compostos fenólicos;
- Obter hidrolisados de isolados da proteína de soro de leite e do girassol através da digestão *in vitro* por INFOGEST;
- Caracterização da hidrólise: avaliação do grau de hidrólise, da massa molecular dos digeridos;
- Padronização do cultivo das células Caco-2 em placas transwell;
- Estudo de transporte transepitelial em células Caco-2 com diferentes concentrações dos hidrolisados e comparação entre matriz vegetal e animal;
- Identificação das sequências peptídicas e aminoácidos totais absorvidas pela barreira gastrointestinal;
- Análise *in sílico* para avaliação do potencial bioativo.

3 REVISÃO BIBLIOGRÁFICA

3.1 Peptídeos bioativos

Os peptídeos bioativos são definidos como fragmentos da sequência de aminoácidos de uma proteína, que são bioativos apenas quando liberados da estrutura proteica original. São moléculas que apresentam uma estrutura menor em comparação com as proteínas e desempenham diversas funções fisiológicas e/ou biológicas a depender da sequência e composição de aminoácidos (Fernández-Tomé & Hernández-Ledesma, 2020) .

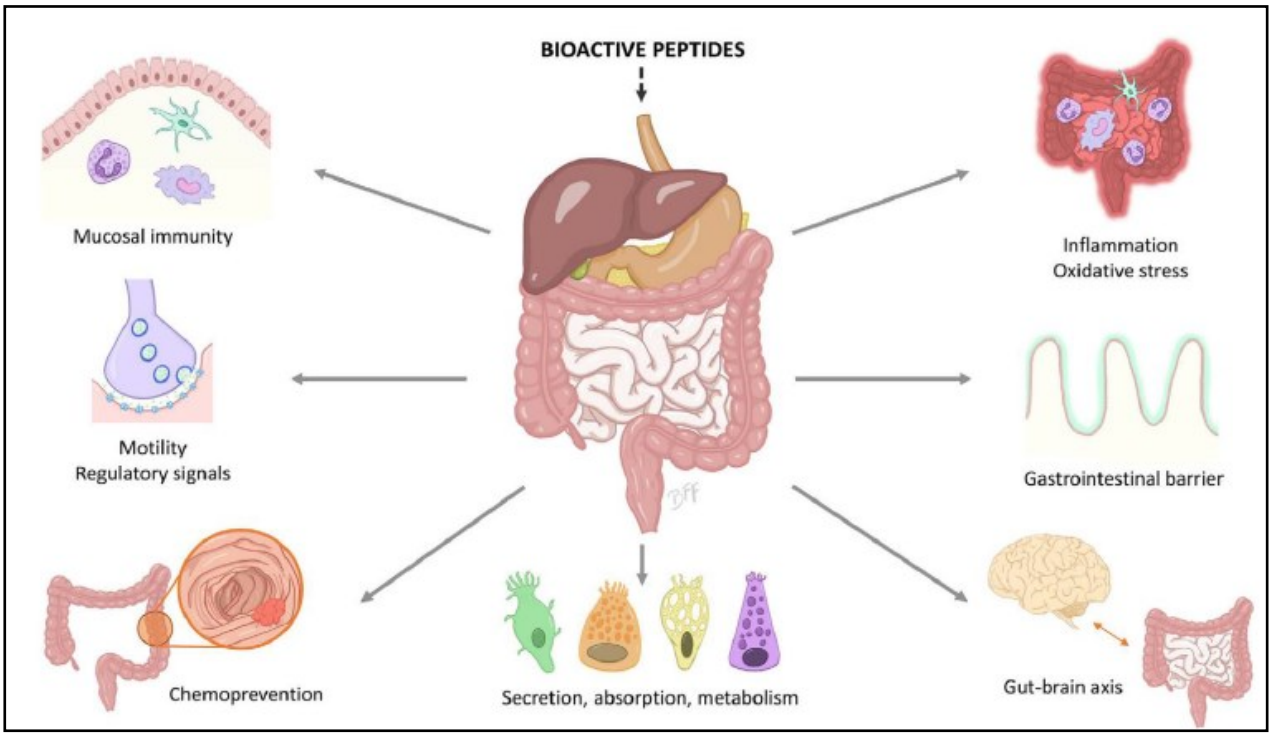

Figura 1. Ilustração das diferentes funções no organismo desempenhadas por peptídeos bioativos por meio da interação com o sistema digestivo.
Fonte: Fernández-Tomé; Hernández-Ledesma (2020).

Nos últimos anos, pesquisas envolvendo peptídeos bioativos derivados de alimentos, cresceram de forma exponencial devido a sua atividade funcional, uma vez que essas atividades podem proporcionar efeitos modulatórios sobre diversas funções fisiológicas no organismo (Figura 1). Os peptídeos alimentares demonstram afetar positivamente os principais sistemas no nosso organismo, tais como: digestivo, cardiovascular, endócrino e nervoso. Os seus efeitos podem estar associados às suas propriedades na absorção intestinal, secreção e metabolismo de nutrientes podendo desempenhar funções imunológica,

antioxidantes, anti-inflamatória e inclusive reguladores, tornando-se relevantes para uso em alimentos funcionais e nutracêuticos (Fernández-Tomé & Hernández-Ledesma, 2020).

Quimicamente, os peptídeos são formados por hidrólise, fermentação ou digestão de proteínas. Os diferentes métodos de processamento (enzimático, térmico, fermentativo, digestivo, etc.) podem mudar as propriedades do peptídeo formado e sua função (Lemes et al., 2016).

3.2 Bioacessibilidade e biodisponibilidade

Estudos têm demonstrado que a quantidade total de um determinado composto de um alimento não é totalmente absorvido e metabolizado por nosso organismo. Isto pode estar relacionado a alguns fatores tais como, a variabilidade interindividual e ao estado de saúde do indivíduo, bem como a forma química do nutriente consumido ou até mesmo devido as característica química da matriz onde o composto se encontra, uma vez que a presença de variados compostos nos alimentos pode colaborar ou reduzir os valores nutricionais. Assim, para avaliar o potencial de absorção de um nutriente pelo o organismo surge a necessidade de destacar dois termos importantes: bioacessibilidade e biodisponibilidade (Lacerda Sanches et al., 2020).A bioacessibilidade pode ser definida como a quantidade de um composto que é liberado por meio de uma matriz alimentar, e que se torna potencialmente disponível para ser absorvido (Carbonell-Capella et al., 2014). Enquanto que a biodisponibilidade refere-se à quantidade de um composto que foi absorvido pelo trato gastrointestinal e está disponível para ser metabolizado por vias normais de absorção. No caso dos peptídeos bioativos, o intestino é o local onde processa-se muitas das suas funções, entretanto, certas bioatividades só podem ser desempenhadas após os peptídeos terem passado pelo processo de absorção por meio dos enterócitos e atingirem os tecidos alvos (Liang et al., 2022).

À vista disso, compreender sobre a importância dos estudos de bioacessibilidade e de biodisponibilidade é essencial para o avanço do mercado na área de produção de fármacos e principalmente para o comércio de alimentos pois, a partir da matriz estudada, pode-se indicar as contribuições dos nutrientes

para o nosso organismo. Levando em consideração que, nem todos os teores totais dos nutrientes inorgânicos presentes nos alimentos estarão bioacessíveis para serem absorvidos pelo corpo.

Nesse sentido, nos últimos anos têm surgido várias técnicas que permitem avaliar tanto a bioacessibilidade quanto a biodisponibilidade de uma determinada substância. Podendo destacar os estudos *in vitro* que, desempenham um papel importante para estimar o impacto da digestão gastrointestinal e das matrizes alimentares (bioacessibilidade), avaliação de compostos fenólicos e potencial funcional de peptídeos resistentes a digestão gastrointestinal (biodisponibilidade) (Gonçalves et al., 2019).

3.3 Digestão gastrointestinal

Compreender quais os compostos dos alimentos são capazes de resistir ao processo de digestão gastrointestinal e serem absorvidos, tem sido amplamente estudado na área da farmacologia, bioquímica e nutrição já que, nos últimos anos tem se notado as exigências do mercado consumidor quanto a qualidade nutricional dos produtos alimentícios. Além disso, entender o processo digestivo é um passo importante para evitar o surgimento de doenças inflamatórias provocas por certos compostos dos alimentos (Brodkorb et al., 2019).

Segundo (Sherwood, 2016), define-se como digestão gastrointestinal, a “quebra bioquímica de componentes alimentares estruturalmente complexos da dieta em componentes menores e absorvíveis pelas enzimas produzidas no sistema digestivo”. Isto é, um processo que altera fisicamente e quimicamente os alimentos onde os nutrientes oriundos do processo digestivo são absorvidos pelo organismo.

O processo de digestão humano tem como principal função, realizar o transporte e a transferência de nutrientes originários de alimentos até os órgãos-alvo. Este processo tem início na boca e finaliza no intestino. É na boca que ocorre a formação do bolo alimentar por meio da quebra mecânica (mastigação) dos alimentos. No ato desse processo, ocorre a secreção de saliva onde, o amido e os demais polissacarídeos são reduzidos a moléculas menores e consequentemente, digeridos por meio da enzima amilase, produzidas durante o processo de salivação (Harper, 1951).

A atuação da enzima amilase é limitada pois quando é exposta à acidez estomacal, a sua ação é inativada. Logo, o bolo alimentar formado é impulsionado para o estômago por meio do processo de deglutição e ao chegar no estômago, o bolo alimentar é armazenado e misturado com o suco gástrico, sendo este formado em sua maioria por ácido clorídrico. Essa mistura permite a ação da enzima pepsina, secretada pelo suco gástrico, que tem a função de degradar os aminoácidos das proteínas, originando peptídeos (Harper, 1951).

Este processo resulta na digestão química do bolo alimentar formando-se o quimo/suco alimentar. Por fim, os componentes da matriz alimentícia são fragmentos e essa ação, já iniciada no processo de formação do suco alimentar, finaliza no intestino. A redução dos componentes ocorre com o auxílio da enzima pancreatina e dos sais biliares e juntamente a esse processo, os nutrientes passam a ser absorvidos pelo lúmen gastrointestinal (Harper, 1951).

3.3.1 Digestão das proteínas

O processo de digestão das proteínas só terá início na etapa gástrica, que ocorre no estômago. A ação do ácido clorídrico (HCl) presente no suco gástrico modifica o pH, que passa de alcalino para ácido podendo ser inferior a 3, permitindo assim a desnaturação das estruturas das proteínas. O ácido clorídrico também é responsável pela transformação do pepsinogênio em pepsina. Essa enzima, na sua forma ativa, cataliza a reação sendo responsável por realizar a hidrólise das ligações peptídicas, assim, as proteínas passam a ser digeridas (Gropper et al., 2018).

Por conseguinte a mistura resultante da etapa gástrica passa para o intestino delgado. Nessa etapa, as proteínas chegam parcialmente digeridas e por meio da ação dos hormônios reguladores (secretina e colecitocinina) o processo de digestão das proteínas é facilitado (Gropper et al., 2018).No fígado é produzido a bile e essa desempenha um papel importante para a digestão de gorduras. A bile possui ação emulsificante, tornando as moléculas maiores de gorduras em pequenas gotículas o que favorece a ação da lipase (Amigo & Hernández-Ledesma, 2020).

É importante ressaltar que na etapa intestinal o pH ácido é modificado por meio da secreção pancreática. O bicarbonato presente na secreção, é um composto alcalino e ao entrar em contato com o quimo torna-o neutro. A ação

do pH é importante para que ocorra a ação das enzimas pancreáticas (proteases, amilases e lipases) e intestinais, pois essas enzimas ao serem liberadas são responsáveis por completar a digestão de proteínas (Amigo & Hernández-Ledesma, 2020).

Ao final, as macromoléculas de proteínas são transformadas em compostos menores e mais simples (peptídeos), sendo mais assimiláveis pelo organismo. Uma vez digeridos, os compostos liberados passam a ser disponíveis para serem absorvidos através dos enterócitos das vilosidades por meio dos mecanismos de transporte (Amigo & Hernández-Ledesma, 2020) .

3.3.2 Fatores que afetam a digestão das proteínas

A digestibilidade, combinada com a composição de aminoácidos essenciais e quantidade proteica, é considerada o fator mais determinante do valor nutritivo de uma fonte de proteína (Loveday, 2023). Entretanto, esta digestibilidade pode ser afetada por fatores endógenos e exógenos. Os fatores endógenos estão relacionados as características estruturais da proteína tais como, alterações na estrutura secundária, ligações cruzadas e hidrofobicidade. Já os fatores exógenos estão relacionados a presença de fatores antinutricionais como os fitatos, taninos, inibidores de proteases, polifenóis, fibras e hemaglutininas (lectinas) (Sá et al., 2020).

As proteínas de origem vegetal são conhecidas por terem uma menor digestibilidade devido a presença dos fatores antinutricionais (Kaur et al., 2022). (Boye et al., 2012) em seus estudos, relatam que a depender da quantidade consumida. Essas substâncias podem interferir nos processos metabólicos e afetar na absorção dos nutrientes no trato gastrointestinal sendo responsáveis por reduzir o valor nutricional do alimento consumido.

Os procedimentos de isolamento apresentaram um efeito positivo na digestibilidade da proteína vegetal, podendo estar relacionado a uma redução nos níveis de taninos e fitatos. Empregar etapas de processamento para inativar ou reduzir a quantidade de fatores antinutricionais, pode ser uma alternativa para melhorar a qualidade da proteína de uma fonte vegetal (Nielsen et al., 2001)

Além disso, nutricionalmente, as matrizes vegetais são consideradas inferiores às proteínas animais por apresentarem um ou dois aminoácidos essenciais limitantes em sua composição. Em sua maioria, as leguminosas

apresentam baixos níveis de metionina e cisteína (aminoácidos sulfurados), enquanto que alguns cereais tem deficiência de lisina. Entretanto, a deficiência de aminoácidos pode ser resolvida por meio da complementação de proteínas, como as misturas de cereais e leguminosas ou por meio da mistura de proteínas vegetais e animais (Sá et al., 2020).

No caso da matriz animal, essas são consideradas de melhor qualidade por apresentarem elevados teores proteicos, bem como todos os aminoácidos essenciais em sua composição e por suas moléculas permanecem mais íntegra após a digestão e por isso têm maior valor biológico. Logo, em comparação com as fontes vegetais, são consideradas fontes proteicas completas de alta digestibilidade e biodisponibilidade (Kaur et al., 2022).

3.3.3 Passagem de peptídeos pela barreira gastrointestinal

Os peptídeos provenientes de alimentos, podem ser digeridos no trato gastrointestinal (TGI) por meio da ação de várias enzimas ou até mesmo por meio da fermentação microbiana. Esses peptídeos são produzidos no lúmen intestinal e são transportados através da monocamada de células epiteliais até atingirem a corrente sanguínea. Até este tempo, os estudos envolvendo o processo de transporte desses peptídeos pode ocorrer por quatro formas distintas: difusão passiva (1), transcitose (2), transporte paracelular (3) rotas medidas por carreadores (4) (Xu et al., 2019a).

O PepT1, ilustrado na Figura 2, é considerado um carregador de alta capacidade. O transporte dos peptídeos mediado pelos PepT1 ocorre por meio da afinidade onde, se é utilizado frações de prótons entre o lúmen intestinal e as células epiteliais. Os peptídeos realizam ligações entre os prótons (H+), que são então transportados para fora dos enterócitos por meio de trocadores de H+/Na+. Dessa forma, a hidrólise parcial dos peptídeos pode ocorrer dentro das células e/ou na corrente sanguínea (Xu et al., 2019b).

Tratando-se então dos mecanismos de absorção dos peptídeos, se faz necessário ressaltar que no intestino há um revestimento de células epiteliais que formam o que conhecemos como a barreira gastrointestinal, como ilustrado na Figura 2.

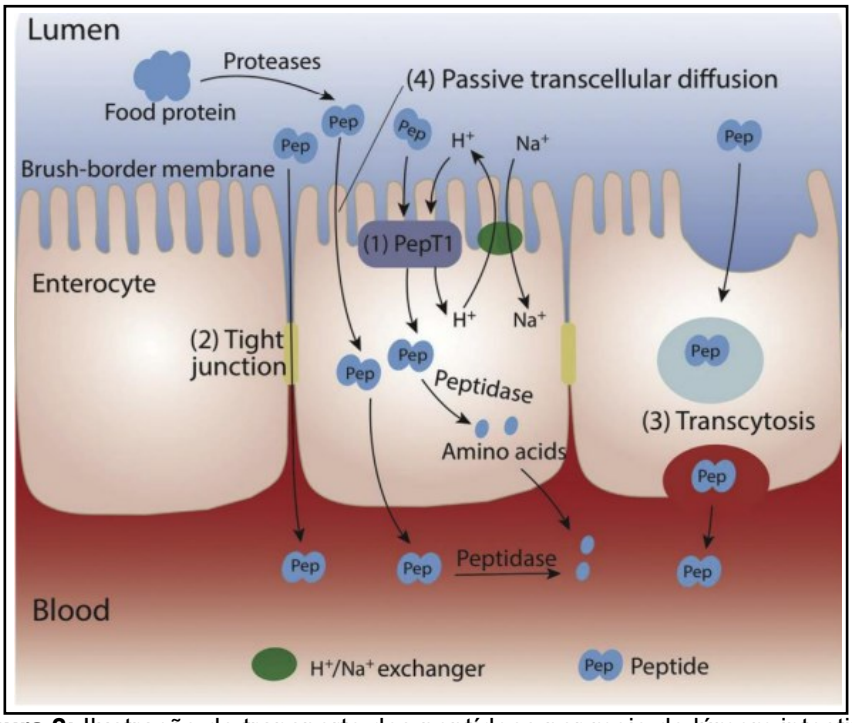

Figura 2: Ilustração do transporte dos peptídeos por meio do lúmem intestinal.

Fonte: Xu *et al*., (2019).

Essas células são conectadas por junções que contribuem para a degradação dos peptídeos e também são responsáveis por controlar a circulação de nutrientes, isto é, realizam o transporte transepitelial que por meio da associação determinam quais compostos serão absorvidos (Liang et al., 2022).

Dentre essa monocamada de células é importante ressaltar os enterócitos, os quais estão em sua maioria. Os enterócitos desempenham um papel importante no destino final dos peptídeos. Morfologicamente, possuem microvilosidades que estão presentes em sua parte superior (apical), sendo comumente denominado de borda em escova, onde essas microvilosidades são responsáveis por aumentar a área da superfície intestinal. Sendo assim, uma vez que os peptídeos atingem o epitélio, eles podem ser transformados, degradados ou absorvidos, dependendo de suas propriedades (Xu et al., 2019b).

É importante ressaltar que, a absorção desses nutrientes está condicionada por diversos fatores como o pH, eliminação pré-sistêmica, já que a quantidade de compostos ao atingir a circulação sanguínea é reduzida por já terem sido excretadas anteriormente no lúmen gastrointestinal e até mesmo por meio das interações com o muco. A camada mucosa consiste em uma

substância viscosa que reveste toda a superfície do tecido epitelial do trato gastrointestinal e tem como funções proteger contra danos mecânicos e facilitar o trânsito gastrointestinal por meio da lubrificação. A sua influência na absorção está associada à sua capacidade de formar ligações iônicas que tem potencial para retardar o processo (Gonçalves et al., 2019; Barthe et al., 1999;).

Na área de alimentos, a técnica de membranas semipermeáveis tem sido amplamente utilizada em paralelo com o método *in vitro* para estudos de transporte transepitelial, sendo fundamental para estudar a passagem de compostos que resistiram ao processo de digestão por meio da difusão passiva. A técnica consiste na colocação da matriz alimentar digerida *in vitro*, em contato com os poços que contêm as membranas e que irão funcionar como um filtro, permitindo somente a passagem de compostos de até 12 kDa (Gonçalves et al., 2019; Miller et al., 1981).

3.3.4 Destino dos peptídeos não absorvidos

Os peptídeos que não são absorvidos pelo organismo seguem diferentes destinos no trato gastrointestinal, isso vai depender muito de suas características químicas e da microbiota intestinal. No geral, eles podem ser degradados, fermentados ou eliminados (Silva et al., 2002).

No primeiro momento, eles podem ser reabsorvidos por células intestinais devido a degradação das enzimas digestivas. No nosso intestino delgado há enzimas digestivas, como as peptidases e proteases, que podem degradar os peptídeos não absorvidos a aminoácidos ou em peptídeos menores facilitando a sua metabolização (Frenhani & Burini, 1999).

Com relação a peptídeos maiores, geralmente esses não conseguem atravessar a membrana mucosa por difusão passiva devido as suas características físico-químicas uma vez que peptídeos menores e de baixo peso molecular apresentam uma maior probabilidade de serem absorvidos. Nesses casos, podem ser absorvidos por mecanismos como pinocitose nos enterócitos ou através das células microfold, embora essa via seja limitada devido à pequena área de superfície disponível (Silva et al., 2002).

Ainda, ao chegarem no cólon, podem ser fermentados por bactérias intestinais. Essa interferência pode resultar na produção de ácidos graxos de cadeia curta, que são benéficos para a saúde intestinal, além de outros

compostos como amônia e compostos fenólicos. Esses subprodutos podem ser usados por bactérias colônicas para fornecimento de energia ou propriedades de novos aminoácidos. Por fim, peptídeos que não são digeridos ou fermentados acabam sendo excretados nas fezes (Frenhani & Burini, 1999).

De forma geral, mesmo sem serem absorvidos os peptídeos podem exercer efeitos benéficos no organismo, podendo modular a microbiota intestinal, fortalecer a barreira gastrointestinal ou interagir com celular receptoras, influenciando a saúde digestiva (Silva et al., 2002).

3.3.5 INFOGEST – protocolo de simulação in vitro estática da digestão gastrointestinal de alimentos

Atualmente existem vários métodos que são comumente usados para simular o processo de digestão *in vitro* dos alimentos, os quais podem ser classificados como métodos dinâmicos e estáticos. Os modelos dinâmicos, em sua maioria, têm se mostrado adequado para simular as condições fisiológicas gastrointestinais envolvendo alimentos e produtos farmacêuticos. No entanto, esses modelos são relativamente caros e complexos, podendo não estar disponíveis para boa parte dos pesquisadores que estudam na área (Brodkorb et al., 2019).

Produzido pela COST INFOGEST, o protocolo padronizado INFOGEST é considerado o método mais simples para simular o processo de digestão de forma estática além de apresentar boa reprodutibilidade, facilidade para realizar avaliação em cada etapa da digestão e com custo relativamente baixo. A amostra de alimento a ser analisada é submetida de forma sequencial a cada etapa da digestão (oral, gástrica e intestinal) em condições fisiológicas já pré-estabelecidas tais como, atividade enzimática, pH, tempo e temperatura de acordo com cada etapa conforme está ilustrado na Figura 3 (Brodkorb et al., 2019).

A fase oral deve ser incluída em todos os procedimentos de digestão simulada, independentemente se o alimento é líquido ou sólido. pois é durante essa fase que o alimento será diluído com o fluido salivar simulado (SSF). Nessa fase, a amilase salivar só será adicionada se a matriz tiver amido em sua composição. Para os alimentos sólidos, é necessário simular a mastigação, ou seja, o alimento deve ser reduzido de tamanhos (picado ou triturado). Após isso

a amostra diluída (que simula a formação do bolo alimentar) é incubada a 37°C por 2 minutos (tempo médio de uma mastigação fisiológica normal) em pH 7 (Brodkorb et al., 2019).

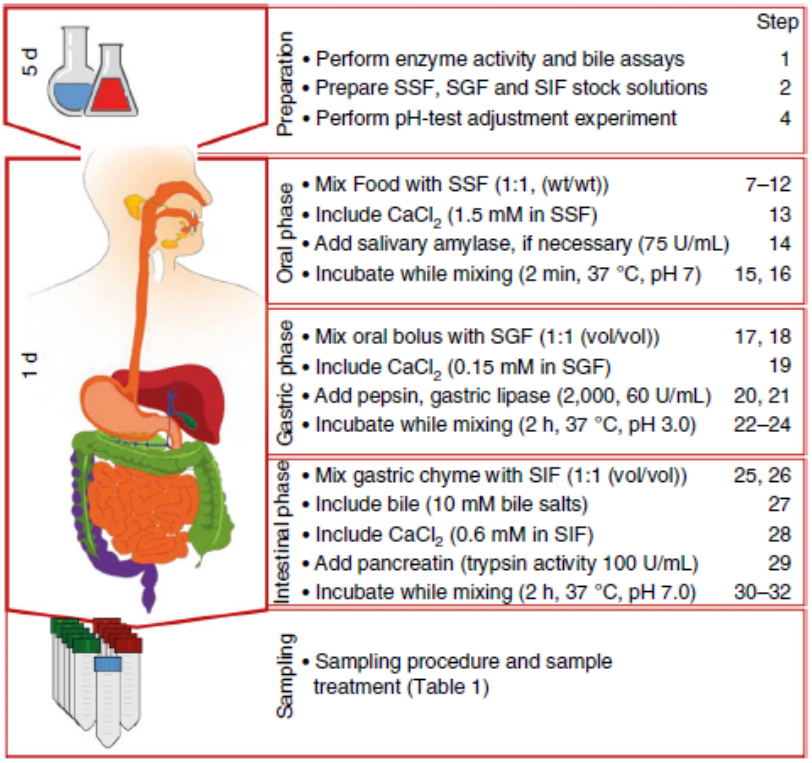

Figura 1: Ilustração das etapas do método de digestão INFOGEST.

Fonte: Brodkorb et al., (2019).

A fase gástrica consiste na mistura do bolo alimentar formado com o fluido gástrico simulado (SGF), mais a adição de enzima gástrica (pepsina). Durante essa etapa é necessário realizar o ajuste do pH de alcalino para ácido para que a enzima possa atuar. Assim, a amostra será incubada sob agitação em pH 3,0 por 2h (baseado no tempo médio que o alimento permanece no nosso estômago). O resultado dessa etapa é a formação do quimo alimentar. É importante ressaltar que para o ajuste do pH pode se utilizar o ácido clorídrico (HCl) e o hidróxido de sódio (NaOH) para básico (Brodkorb et al., 2019).

Na última fase (intestinal), o quimo gástrico formado é então diluído com o fluido intestinal simulado (SIF), mais adição de sais biliares e enzimas pancreáticas sendo esta última baseada na atividade da tripsina. Caso seja utilizada enzimas de forma individual, é necessário fazer a adição de todas as substâncias: tripsina, quimotripsina, lipase pancreática, colipase com lipase,

amilase pancreática e bile. Por conseguinte, é realizado a correção do pH para 7 e a diluição é incubada a 37°C por 2h (Brodkorb et al., 2019).

Ao finalizar a fase intestinal, deve-se interromper a ação da protease por meio do congelamento rápido ou inibidor de protease. A amostra deve então, ser centrifugada, o sobrenadante é retirado e o precipitado é armazenado sob resfriamento. Após isso, a amostra é liofilizada, estando pronta para analises posteriores. O protocolo pode ser usado para avaliar os produtos finais resultantes da digestão de alimentos, bem como a liberação de micronutrientes como por exemplo, peptídeos/aminoácidos, ácidos graxos, açúcares simples e entre outros (Brodkorb et al., 2019).

Além disso, a técnica não permite a representação total e exata do processo de digestão, pois o processo digestivo humano é complexo e muitos fatores presente no organismo (variação de pH, genética, concentrações de enzimas, tempo e etc.) podem interferir, já que a digestão humana ocorrer de forma gradual e não sequencial. Apesar disso, o modelo tem mostrado resultados bastante eficientes para os estudos de simulação digestiva e por ser um modelo padronizado é possível realizar comparações, os quais geram resultados mais autênticos (Hur et al., 2011).

3.4 Modelo de absorção *in vitro*

O uso da técnica *in vitro* frequentemente está associado ao uso da técnica *in vivo*, não por serem similares, mas por ser uma técnica com potencial para substituição de experimentos em animais. Dentro do estudo de verificação da bioacessibilidade e a biodisponibilidade de um determinado composto, os testes *in vivo* estão mais relacionados a estudos de balanço de massa, como por exemplo nas análises de compostos fenólicos. O objetivo é determinar a quantidade de uma substância absorvida através da diferença entre as quantidades ingeridas e as quantidades excretadas pelo animal. Entretanto, as análises *in vivo* têm o fator genético e características morfológicas bastante relevantes, já que se diferencia da fisiologia humana gerando um grande impacto na confiabilidade do resultado final (Alminger et al., 2014; Neto et al., 2017).

Em contrapartida, as técnicas *in vitro* permitem avaliar a biodisponibilidade de determinados compostos de uma forma mais fundamentada já que se baseiam na fisiologia humana. Além disso, os modelos

in vitro são mais econômicos, rápidos, práticos e reprodutíveis para entender e avaliar as características de permeabilidade de diferentes compostos alimentares, além de serem mais éticos (Hur et al., 2011).

3.4.1 Linhagem de cultura celular Caco-2

O emprego de linhagens celulares está associado ao modelo de digestão simulada in vitro e dentre as linhagens de culturas celulares mais utilizadas estão as células Caco-2. São pertencentes a uma linhagem de células de um adenocarcinoma de cólon humano e possuem a capacidade de se diferenciar em fenótipo similar ao de um enterócito humano maduro, apresentando muitas de suas propriedades morfológicas e funcionais (Natoli et al., 2012).

Por apresentarem tais características e por serem cultivadas em monocamadas torna-se possível desenvolver estudos sobre a absorção dos compostos de interesse. No campo dos peptídeos bioativos, por exemplo, tem se notado o crescente avanço da aplicação dessas células atreladas a técnica *in vitro* para avaliar a absorção dos peptídeos e os mecanismos que envolve o transporte desses através das monocamadas de células Caco-2 (Amigo & Hernández-Ledesma, 2020).

Os estudos de simulação do lúmen gastrointestinal, envolve a utilização de placas com inserto que contêm uma membrana porosa semipermeável com dois lados. Dessa forma, cada poço fica com um compartimento superior (apical) e um inferior (basolateral) contendo meio nutritivo que retrata a circulação sanguínea. As células confluentes, ficam então aderidas na membrana porosa que separa os compartimentos, sendo então possível simular o mecanismo de transporte dos peptídeos no intestino (Amigo & Hernández-Ledesma, 2020).

A utilização de células Caco-2 apresenta como principal vantagem o fato de derivarem de uma linhagem celular humana, ou seja, não há a diferenciação fisiológica e morfológicas entre as espécies. Além disso, em comparação com outros modelos celulares ela apresentam uma rápida diferenciação, são cerca de 21 dias de cultivos para expressarem as propriedades morfológicas dos enterócitos maduros do intestino delgado (Panse & Gerk, 2022).

Em contrapartida, o intestino humano contém mais do que um tipo de células, não apenas enterócitos, enquanto a linha celular Caco-2 contém exclusivamente enterócitos. Foi demonstrando também que, o nível de

expressão das enzimas na borda em escova das células caco-2 é menor em comparação com o intestino humano, isso impacta no metabolismo dos peptídeos que uma vez cultivados com essas linhagens celulares podem ser metabolizados em menor quantidade ou abaixo do seu limite de detecção (Lea, 2015). Além disso, essas células se caracterizam pela ausência de uma camada protetora de muco o que pode afetar no transporte de peptídeos e aminoácidos, uma vez que o muco influência na modulação da absorção de nutrientes (Panse & Gerk, 2022).

Ainda, advém ressaltar que se trata de uma linhagem celular proveniente de um carcinoma. Embora sejam geralmente aceitas devido a sua praticidade, é necessário desenvolver mais estudos para a aplicação dos peptídeos uma vez que as células Cacos-2 podem desempenhar ou possuir propriedades distintas das células normais (Panse & Gerk, 2022).

3.5 Proteína de origem animal - Soro de leite

3.5.1 Aspectos gerais

O soro do leite bovino é um dos produtos alimentícios mais utilizado como objeto de estudo por ser rico em proteínas, sendo essas conceituadas como proteínas de alta qualidade por apresentarem em sua composição cadeias de aminoácidos bem balanceadas em relação a aminoácidos essenciais e não essenciais podendo então, serem avaliadas quanto ao seu potencial benéfico a saúde (Brodkorb et al., 2019). Estas proteínas não apresentam restrições etárias pois atendem às recomendações da Food and Agriculture Organization para todas as idades (Nutrition Division, 1991).

Por constituírem um grupo bastante diversificado de proteínas com características heterogêneas, as proteínas do soro do leite são as mais empregadas em formulações alimentícias (Kazmierski & Corredig, 2003). Suas principais características estão relacionadas ao seu valor biológico elevado, rápida absorção pelo organismo e elevada digestibilidade. As principais proteínas do soro do leite são α-lactoalbumina (α-La), β-lactoglobulina (β-Lg), e albumina de soro bovino (BSA) sendo que, as duas primeiras constituem entre 70 a 80% das proteínas totais do soro e suas concentrações no leite bovino são 1,2g/L, 3,2 g/L, e 0,4 g/L, respectivamente (Sgarbieri, 2004).

3.5.2 Hidrólise e bioatividade

Quando submetidas a hidrólise, apresentam modificações em suas propriedades químicas, físicas, e funcionais, sem prejudicar seu valor nutritivo. Em vez disso, os peptídeos originados por meio da hidrólise, ao atingirem o intestino delgado, apresentam uma elevada taxa de digestão e absorção e com isso, a concentração aminoacídica do plasma rapidamente aumenta, estimulando a síntese de proteínas nos tecidos. Sendo assim, as proteínas hidrolisadas do soro do leite apresentam melhoras com relação as características de absorção intestinal (Boirie et al., 1997).

A bioatividade dos peptídeos dos hidrolisados do soro do leite tem sido amplamente pesquisada nos últimos anos, no entanto, pouco se sabe sobre quais peptídeos são capazes de resistir às duras condições do trato gastrointestinal e serem absorvidos por nosso organismo (Fernández-Tomé & Hernández-Ledesma, 2020). Estudos recentes empregaram monocamadas diferenciadas de Caco-2 para avaliar essa absorção intestinal (Benedé et al., 2022; de Espindola et al., 2023; Liu et al., 2023; Ren et al., 2025; Takumi et al., 2024), sendo necessário desenvolver mais pesquisas na área para se obter mais informações a respeito da biodisponibilidade desses peptídeos.

3.6 Proteína de origem vegetal – girassol

3.6.1 Aspectos gerais

O girassol, do grego *helios* que significa sol, e *anthus*, que significa flor, ou "flor do sol", é uma planta de cultivo anual, pertencente à família Asteraceae, e de grande importância econômica mundial (Singh et al., 2022). O seu destaque está em suas sementes, que dispõe de baixo teor de fatores antinutricionais e alto teor de gorduras poli-insaturadas, além de apresentar vitaminas do complexo B, minerais, fibras e polifenóis, como o ácido clorogênico. Segundo a Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), o fruto do girassol tem 24% de proteínas e apresenta ainda em sua composição lisina, metionina, cisteína e triptofano como os principais aminoácidos essenciais (de Oliveira Filho & Egea, 2021).

Seu principal uso está associado à extração de óleos onde é considerado o quarto óleo vegetal mais popular e consumindo no mundo sendo utilizado em diversos segmentos de mercado como alimentício, coméstico e industrial. Em comparação com os demais óleos vegetais, o óleo de girassol é de alta qualidade devido a sua alta proporção de ácidos graxos insaturados (Salgado et al., 2011). Segundo a EMBRAPA, o girassol tem 47,3% de óleo, sendo rico em ácido linoleico, essencial para o organismo humano.

Durante o processo de extração do óleo, é gerado o farelo do girassol, um subproduto que pode ser utilizado como uma alternativa para aplicação como ingredientes devido as suas propriedades funcionais (Salgado et al., 2011).

3.6.2 Mercado atual e aplicação

O girassol é uma oleaginosa de importância global, sendo a terceira oleaginosa mais produzida no mundo, a quarta cultura mais importante na produção de óleo vegetal e a terceira cultura mais importante na produção de farelo de oleaginosa; entre outras fontes de proteína. Seus principais produtores são EUA, Ucrânia e Argentina (Dantas et al., 2017). No Brasil, a cultura do girassol encontra seu principal cultivo nos estados de Goiás, Mato Grosso e Minas Gerais, onde as condições climáticas são mais favoráveis (Morpurgo, 2023).

De acordo com a revista Cultivar, o mercado de óleo de girassol está projetado para atingir milhões de dólares até 2028. Essa ascensão está sendo impulsionada principalmente pela expansão da produção de biodiesel e pela crescente demanda por alimentos saudáveis. Nos últimos anos tem-se observado que os consumidores têm exigido cada vez mais produtos alimentícios com alto valor nutricional, efeito benéfico a saúde e livre de aditivos químicos.

A aplicação do subproduto do girassol como um ingrediente para melhorar as farinhas, está relacionado ao seu elevador teor de proteína sendo capaz de desempenhar atividades nutricionais e funcionais nos alimentos. Esta também é uma das causas da aplicação da torta de girassol para enriquecimento em ração animal (González-Pérez & Vereijken, 2007). Na indústria de alimentos, o subproduto é transformado em farelo/farinha para ser utilizado como um

ingrediente, principalmente nos produtos de panificação tais como: massas, biscoitos, pães, barras de cereais e entre outros (de Oliveira Filho & Egea, 2021).

3.6.3 Hidrólise e bioatividade

De fato, os consumidores estão cada vez mais preocupados com problemas ambientais, esgotamento de recursos naturais e impactos no meio ambiente. Sob esse ponto de vista, as agroindústrias geram uma grande quantidade de subprodutos ricos em compostos bioativos com potencial para serem utilizados como matéria-prima para o desenvolvimento de alimentos e materiais funcionais.

O subproduto do girassol pode ser um importante contribuinte para a produção de peptídeos ou aminoácidos que, uma vez liberados da molécula de proteína, podem desempenhar efeitos funcionais no organismo (Lemes et al., 2016).

A literatura informa que os seus hidrolisados demonstraram atividades biológicas importantes na saúde humana, como atividades antioxidantes (do Prado et al., 2021), anti-hipertensivas (Dabbour et al., 2019), bem como efeitos anti-inflamatórios e imunomoduladores (Velliquette et al., 2020).

Sendo assim, pensando nos consumidores que buscam por produtos que adotam o conceito mais equilibrado, o aproveitamento de resíduos surge como uma alternativa para gerar uma indústria mais sustentável. Além disso, a obtenção de proteína a partir da torta do girassol surge como uma alternativa para a substituição de proteínas de origem animal, além de atenderem as exigências do mercado atual que preza por produtos que apresentem contribuições nutricionais.

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4 RESULTADOS

Epithelial gastric-intestinal transport study of whey or sunflower peptide: a comparison of animal and plant protein absorption

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ABSTRACT

Plant-based proteins are sustainable alternatives to animal sources and bioactive peptide may be extracted with functional effect. However, their digestive and absorptive equivalence remains unclear. This study compared the digestion, transport and bioactivity of whey (WPD) and sunflower (SPD) protein isolates. Both reached high hydrolysis (>60%) but yielded distinct peptide profiles: SPD ranged from 0.2–2 kDa, while WPD was concentrated between 0.2–0.4 kDa. Using a Caco-2 transwell model, WPD demonstrated significantly higher transport efficiency (35.2%) and greater resistance to brush-border peptidases than SPD (14.9%). While aromatic and positively charged amino acids showed enhanced transportability, peptide uptake appeared independent of molecular size, suggesting a non-specific mechanism like transcytosis. *In silico* analysis identified diverse bioactive peptides in the basolateral compartment for both sources. Overall, despite both proteins producing functional sequences, WPD exhibits superior bioavailability and peptidase stability. These findings provide insights into nutritional and bioactive differences between animal and plant proteins.

Keywords Bioactive peptides, intestinal absorption, Caco-2 cell model, whey protein isolate, sunflower protein isolate, peptide bioavailability, simulated gastrointestinal digestion

1 INTRODUCTION

There is a growing global demand for food proteins due to the increase in population, longer lifespan, as well as because the protein relevance in the diet with beneficial nutritional effect. To meet the new needs of the market, the food industry has innovated in new formulations based on alternative sources of proteins, such as plant, fungi, insects and microbial proteins, with less environmental impact production than traditional meat source and less animal mistreatment.

The biological and physiological activities of proteins are attributed to several peptides that, when released from their original protein structure can also perform various physiological effects, acting as protective molecules against several body dysfunctions (Patel, 2015). However, the bioactive effect of food-derived peptides can only be validated when they resist as integral form to the digestive enzyme, as well as are capable of being absorbed by the gastrointestinal cells to richen the target organ. Nonetheless, the formation of these peptides during the digestive process is little known, especially in relation to plant-based proteins, as well as their absorption capacity.

In this scenario, vegetable plant by-products, particularly sunflower flour, which is derived from oil seed production, can serve as an alternative for the preparation of food products of high functional value, since they have adequate sources of proteins (> 30% in defeated sunflower meal) (Arrutia et al., 2020; Hoehnel et al., 2022). Furthermore, several peptides derived from sunflower seeds, produced during simulated digestion, exhibited antioxidant and anti-inflammatory activity in Caco-2 enteric cells (Tonolo et al., 2024; Lopes et al., 2025) (Lopes et al., 2025; Tonolo et al., 2024). However, little has been explored about the capacity of these peptides to be absorbed and promote functional effect. In contrast, the bioavailability and bioactivity of animal based peptides are better explored. Some sequences of whey peptides formed by simulated digestion process showed transport capacity across Caco-2 intestinal barrier, with potential of acting as hypertensive and antioxidant in muscle cells (Corrochano et al., 2019; Liang et al., 2022).

In general, plant-based proteins present some limited essential amino acids, such as lysine, in the case of sunflower, and present lower digestibility than

animal proteins due to several anti-nutritional factors. However, the processing of plant-based products, such as protein isolation, may improve digestibility (Kaur & Ghoshal, 2022). In addition, the combination of different protein sources may improve the nutritional value of plant-based product. The fermented mixture of sunflower seed cake and whey demonstrated strong degradability of protein after simulated digestion with low molecular weight peptides (<3 kDa), which can contribute to the development of bioactive activities (Mendo et al., 2024).

In vitro simulated gastrointestinal digestion (SGID) assays are useful tools to investigate the interaction and stability of the peptide against digestive enzymes (Segura-Campos et al., 2011). At the same time, new approaches that combine SGID with intestinal cell lines have been used to better investigate the bioavailability of peptides originating from different protein sources and optimize absorption assays. Caco-2 cells are among the most widely used cells as a model for the study of the gastrointestinal barrier (Amigo & Hernández-Ledesma, 2020). This cell line, derived from human colonic adenocarcinoma, under ideal culture conditions, can spontaneously differentiate into polarized structures similar to human enterocytes, with apical brush-border features and microvilli (Xu et al., 2019b).

The study of the digestion and bioavailability of amino acids and peptides from plant and animal protein sources has become important for assessing the impact of dietary variation on new food products. One way of studying this is to compare the digestion and absorption of amino acids and peptides between plant and animal sources. Therefore, the aim of this study was to investigate the bioacessibility of peptides derived from whey or sunflower protein submitted to SGID and the bioavailability in Caco-2 cells, grown on transwell membrane, which mimic the transit of peptides across the gastrointestinal barrier. The degree of hydrolysis and molecular weight distribution of hydrolysates was evaluated after digestion. The peptides from sunflower or whey hydrolysates present in the apical and basolateral chamber were sequenced, identified by their protein origin and compared according to the rate of transportability across cell monolayer and peptide integrity. The biological functionality was related to peptide structural aspects, such as hydrophobicity, molecular weight and cross-reference with bioactive database.

2 MATERIALS AND METHODS

2.1 Chemicals

To quantify the phenolic compounds, sodium carbonate, gallic acid, obtained from Sigma Aldrich (St. Louis, USA) was used. Folin-Ciocalteu and ethanol (99.5%) were purchased from Dinâmica (São Paulo, Brazil). The methanol (99.9%, HPLC grade) was obtained from JT Baker (Bridgewater). Reagents, OPA (P1378), sodium tetraborate, sodium dodecyl sulfate or SDS, DL-dithiothreitol or DTT (S43819), as well as digestive enzymes (pepsin and pancreatin) and standards, α-lactalbumin (L6385), insulin (I2643), vitamin B12 (V2876), 3,4-dihydroxy-L-phenylalanine (D9628), aprotinin (A1153-1MG), DL-2-aminobutyric acid or AAAB (162663), phenyl isothiocyanate (PITC - P1034) and bovine serum albumin (A4503), were obtained from Sigma Aldrich (St. Louis, USA). The amino acid (P/N20088, H Pattern) and L-Serine (36323) standards were obtained from Pierce Biotechnology (Massachusetts, USA). For *in vitro* analysis with cell culture, phosphate-buffered saline (PBS) and Dulbecco-modified Eagle medium (DMEM) were purchased from Gibco (Waltham, MA). Fetal bovine serum (FBS), EDTA −10 mM trypsin solution, non-essential amino acids, and L-glutamine were purchased from Vitrocell Embriolife (Campinas-SP, Brazil). Sodium pyruvate solution, Hank's balanced salts solution (HBSS), thiazolyl blue tetrazolium bromide (MTT), and a solution containing 10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin, obtained from Sigma Aldrich (St. Louis, USA). Lucifer Yellow (LY) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). 12-well transwell cell culture flasks (1.13 cm^2 and 0.4 µm pore size) were purchased from Greiner Bio-One (Monroe, NC, USA) and the water was Milli-Q ultrapure (Merck Millipore, Darmstadt, Germany). Other reagents were used in the analytical class. The analyses were performed in triplicate.

2.2 Samples

Commercial whey protein isolate (WPI) was purchased from Glanbia Nutritionals. Sunflower meal (H. annuus L.) was supplied and defatted by the fiber laboratory of the Center for Food Science and Quality (Institute of Food

Technology – ITAL, Campinas-SP). The residual oil was extracted by supercritical CO2, where 90% of this compound was reduced (from 34.98 to 3.53 g/100g).

2.2.1 Reduction of phenolics from defeated sunflower meal

Defatted sunflower meal (DSM) was submitted to phenolic extraction based on the methodology of Salgado *et al*., (2011). For this, 70% ethanol (v/v) was used as extraction solvent in the proportion (1:15 p/v) at pH 5 (HCL 1 mol/L) under agitation. After 1 hour, the same initial volume of 70% ethanol was added to promote reextraction to ensure the reduction of phenolic content (under the same conditions) followed by centrifugation (11000xg for 20 min at 20°C). The supernatant was discarded (phenolic compound + alcohol) and the precipitate (fibers and proteins) was reserved.

2.2.2 *Sunflower Protein Isolate (SPI)*

The protein insulation was made by isoelectric precipitation according to Salgado et al., (2011) with some adaptation. Initially, the precipitate (proteins and fibers) was dispersed in distilled water (5 mL) and the pH was adjusted to 9 (NaOH 1 mol/L). The sample was shaken for 1 hour with pH-metry Frequently of centrifugation (11000×g for 20 min at 20ºC). In the second extraction, isoelectric precipitation of the proteins was performed, for this, the supernatant had pH adjustment to 4.5 (HCl 1 mol/L) and after 1h followed another centrifugation (11000×g for 20 min at 4ºC). The precipitate was subjected to a third extraction with 70% ethanol (pH 5/NaOH 1 mol/L), this time at night rest. Or The material was centrifuged (11000×g for 20 min at 4ºC), the supernatant discarded and the precipitate dispersed in water (until homogenization) with pH adjustment to 7 (NaOH 1 mol/L) followed by freezing and freeze-drying.

2.3 INFOGEST: A standardized model of static gastrointestinal digestion in vitro

The in vitro *digestion process* followed the static protocol INFOGEST 2.0 (Brodkorb et al., 2019).The three phases of the digestion process (oral, gastric and intestinal) were simulated sequentially using all simulated fluids (salivary,

gastric and intestinal) with established physiological parameters (pH, time, enzymes, dilutions, etc.) so that all incubations were carried out at 37°C in a shake bath. Milli-Q water was used to adjust the final volume of each phase. Because these were isolated protein samples, no enzymes were used to digest starch and fat (amylase, bile, and pancreatic lipase).

In summary, the oral phase lasted 2 min. WPI and SPI isolates (1g) were reconstituted in Milli-Q water (5 mL) and mixed with salivary fluid (4mL) with final volume adjustment to 10 mL. The gastric phase had a digestion time of 2 hours. In this phase, the oral bolus was mixed with gastric fluid (8 mL) and pepsin (2000 U/mL), followed by pH adjustment with HCL (1N) to 3 and final volume of the solution to 20 mL. For the intestinal phase, gastric chyme was mixed with simulated intestinal fluid (11 mL) and pancreatin (100 U/mL) diluted in 5 mL of Milli-Q water to achieve the final concentration. The sample had pH adjusted with NaOH (1 N) to 7 and the final volume adjusted to 40 mL. After 2 hours of digestion, the hydrolysates were submitted to a boiling water bath at 85ºC for 10 minutes to ensure the inactivation of the enzymatic activity followed by centrifugation at 7000 g for 20 min at 4ºC. The protein pellet was discarded and the collected supernatant was filtered on a PTFE membrane (0.45 µm) and lyophilized.

2.4 Peptide extraction

To obtain peptides compatible with incubation in cell culture, the samples digested *in vitro* were previously treated as described by Liang et al., (2022) with modifications. Briefly, the dry fraction were purified for in solid phase C18 for elimination of impurities and salts. The crude peptides were resuspended in 2 mL of 0.1% TFA and the same volume of methanol and 100% acetonitrile (ACN) was used to activate the filtration system. The volume of 3 mL of 0.1% TFA was used to balance the column and the peptides were eluted in 1 mL of 60% acetonitrile (ACN). The collected fraction was dried in the nitrogen chamber (5kPa, 40°C for 1h) and stored under freezing.

2.5 Chemical characterization

The samples: Whey Protein Isolate (WPI), Whey Protein Digestion (WPD), Defatted Sunflower Flour (DSM), Sunflower Protein Isolate (SPI) and Sunflower Protein Digestion (SPD)), were characterized by the measurement of total protein content, degree of hydrolysis, molecular size, and total amino acids following the methodology of Latimer, (2012). The determination and phenolic extraction occurred only for the plant sample and followed the methodology described by (Alexandrino et al., 2017).

2.5.1 Extraction and dosing of total phenolics

For the extraction of total polyphenols, methanol was used as an extracting solvent. For this, 0.5 g of lyophilized sunflower protein isolate was mixed with a volume (10 mL) of 80% methanol with Ultra-Turrax T-25 for 1 min followed by centrifugation at 2000 rpm for 10 min. The extraction was repeated three times sequentially and the supernatant, where the extracts were concentrated, was collected in a volumetric flask (50 mL). Aliquots (1 mL) of the extract were removed and mixed with the Folin-Ciocalteu reagent followed by incubation. After 5 min, the volume (10 mL) of the 7% sodium carbonate solution was added. The mixture was incubated for 90 min in the absence of light. Gallic acid solution was used as the standard for the calibration curve (Kim *et al*., 2003). The reading was performed in a spectrophotometer with a wavelength of 750 nm against white and the results were expressed in mg of gallic acid equivalents (GAE) per g of sample. The analyses were done in duplicate.

2.5.2 Protein content and degree of hydrolysis

The proximal protein compositions were determined by the Kjedahl method and to calculate the conversion of nitrogen to protein, a factor of 6.38 for whey protein and 5.75 for sunflower protein was used AOAC, 960.52 (Latimer, 2012).

The degree of hydrolysis (GH) was based on the reaction of the amino group with the o-phthaldehyde reagent (OPA) in the presence of dithiothreitol (DTT). L-serine was used as the standard (Pierce Biotechnology, Illinois, USA). To calculate the degree of hydrolysis of the samples, Eq. (1)

(1) $$\%DH = B * Nb * \frac{1}{\alpha * \mathrm{MP}} * \frac{h}{h(t)} * 100\%$$

Where

%DH = Degree of hydrolysis;

B = Base intake (mL);

Nb = Normality of the base;

α = Average degree of dissociation of the α-NH groups;

PM = Protein mass (g);

h = n° of peptide bonds in the sample

h(t) = Total number of peptide bonds on the protein substrate (meqv/g protein). Whey protein h(t) = 8.88 and sunflower protein h(t) = 7.8.

2.5.3 Molecular weight distribution (Mw)

The separation of peptide fractions by molecular weight range was performed by Rapid Size Exclusion Protein Liquid Chromatography (SE-FPLC) (Akta Pure Chromatograph, GE Healthcare) with gel columns (Superdex 200 and Superdex 30 models) equipped with a UV detector (280 nm). The samples (5 mg protein/mL) and standards (1 mg/mL) were solubilized in sodium phosphate buffer (25 mM, pH 7.4 with 150 mM NaCl). Prior to injection, the samples were filtered on a PTFE membrane (0.45 μm) and sounded for 10 min. After injection, the isocratic flow of the samples was monitored (0.5 mL/min for 120 min at 280 nm). In the end, their molecular weights were determined by comparison with the retention times of the standards used, α-Lactalbumin (14178 Da), Insulin (5807.6 Da), Vitamin B12 (1355.4 Da), L-β-4-Dihydroxyphenylianan ine (197.2 Da) were used at a concentration of 1 mg/mL (de Espindola et al., 2023).

2.6 Cell culture

Human colorectal adenocarcinoma cell lines, Caco-2 (Rio de Janeiro Cell Bank – BCRJ, Rio de Janeiro, Brazil) were cultured in 75 cm2 culture flasks with Dulbecco-modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 8.4 mM Hepes, 23.8 mM NaHCO3, 1% sodium pyruvate, L-glutamine, 1% non-essential amino acids, 100 U mL−1 penicillin, and 100 μg mL−1 streptomycin (Sigma Aldrich, St. Louis, USA). Incubated in an atmosphere of 5% CO2 and 37 °C with medium replaced every 48 h. The number of approved was

between 38 and 42. When 80% confluency was reached, the cells were subcultured after treatment with trypsin-EDTA solution.

2.7 Toxicity test

For feasibility experiments, Caco-2 cells were seeded at a density of 3 × 105 cells cm $^{-2}$ in 24-well plates and incubated for 48 h at 37 °C. After confluency, the medium was removed and the cells were exposed for 2 h to various concentrations of the hydrolysates (0.01, 0.05, 0.1, 0.5, 1.0 and 2.0 mg/mL) diluted in DMEM without supplemented serum and subsequently incubated with MTT (*(3-(4,5-Dimethylthiazole-2-yl)-2,5-iphenyltetrazolium bromide)* at 5 µg mL $^{-1}$ in phosphate-buffered saline solution (PBS) added to the wells for 30 minutes before the end of treatment. After the incubation period, the medium was removed and the volume of 300 µL of dimethyl sulfoxide solution (DMSO, Sigma-Aldrich, St. Louis, Missouri, USA) was added to the wells to dissolve the formazan crystals formed during the treatment. Absorbance was measured at wavelengths of 560 nm and 650 nm in a microplate reader. Cell viability was calculated as a percentage relative to the control (untreated cells).

2.8 Caco-2 monolayer integrity and transport study

The transport of transepithelial peptides was carried out according to the method described by Liang et al., (2022) with adaptations. Caco-2 cells were seeded (1×105 cells/cm2) in 12-well filter support inserts with polyethylene terephthalate membranes (1.13 cm² and pore size 0.4 µm). Before starting the transepithelial transport experiments, the cells were cultured for 21 days or until spontaneous differentiation was allowed.

During the cultivation period, the integrity of the cell monolayers was assessed by transepithelial electrical resistance (TEER) measurements using a Millicell® ERS voltmeter (EMD Millipore, Eschborn, Germany). The measurement was made at three different points on the inserts. The transport study was only started when the monolayers reached TEER values higher than 250 Ω cm². On the day of treatment, TEER was evaluated and recorded, then the Caco-2 cell monolayers were gently rinsed twice with Hank's balanced saline solution (HBSS) and incubated for 15 min in HBSS at 37°C.

After the acclimatization period, the buffer was removed and a volume of 500 µL of HBSS transport medium supplemented with the hydrolysates (WPD and SPD) was added on the apical side (AP) and 1500 µL of HBSS added on the basal side (BL). At the end of 2 hours of treatment, the solutions of the AP and BL compartments were collected separately in the volume of 500 µL, identified in microtubes, lyophilized and stored under freezing for peptide analysis. Control was performed only with HBSS without hydrolyzed supplementation.

2.8.1 Integrity of the cell monolayer with Yellow Lucifer

After treatment with the hydrolysates, the paracellular permeability assay was performed using the Lucifer Yellow marker (LY) to evaluate the integrity of the cell monolayer, as described by Ferraretto et al., (2018).

Subsequently, the volume of 500 µL of LY solution (0.1 mg/mL) in transport medium (HBSS) and 1500 µL of HBSS were added to the AP and BL side, respectively. After incubation for 1 h, compartmental solutions were collected and fluorescence (e.g., 485 nm/in: 535 nm) was measured. In addition, the fluorescence of HBSS buffer (white) and Lucifer Yellow Solution 0.1 mg/ml (control) were measured. The percentage of LY permeability on the basolateral side was determined using Eq. (2):

$$\%\,\mathbf{permeabilidade} = \frac{\mathbf{amostra - branco}}{\mathbf{Ly - Branco}}\ \mathbf{x\ 100}$$

A permeability <3% is acceptable (Sigma-Aldrich, Catalog Number MTOX1000P24).

2.9 Determination of total amino acids

The amino acid profile was evaluated only in the AP and BL compartments of the treated cells (subsection Transepithelial Transport Studies). The release of the individual amino acids occurred by acid hydrolysis using a hydrochloric acid solution (6 N) for 22 h in a digester block at 120 °C. Next, a pre-column derivatization with phenylisothiocyanate (PITC) was performed to recover the hydrolyzed amino acids. For separation, the volume (50µl) was injected into a Luna-Phenomenex C18 reversed-column reversed-phase chromatograph (4.6 mm × 250 mm; particle size 5 µm) (Phenomenex Inc., Torrance, USA) equipped

with a diode array detector (DAD) (Shimadzu, Brazil) at 254 nm. The mobile phase consisted of eluents A and B with sodium acetate, Acetonitrile (99.7-100%, HPLC grade), ultrapure water and disodium EDTA. The identification of the peaks of the compounds was performed by comparing them with the retention times obtained with the external standard (Pierce, PN 20088), the internal standard (α-aminobutyric acid, AAAB) was used in the quantification and the results consisted of the comparison between the digested fractions with the absorbed fractions. Tryptophan has not been analyzed.

2.10 Peptide identification with Liquid Chromatography - Mass Spectrometry (UPLC-MS-MS)

Initially, the dry samples of PA and BL were previously treated. For this, reversed-phase chromatography was used using a C18 matrix device and buffer to desalinate the samples (as described in Section 2.4). Subsequently, the peptides were analyzed with Orbitrap Fusion Lumos Mass Spectrometer (Thermo Scientific) connected with Liquid chromatograph Ultimate 3000 RSLCnano (Thermo Scientific). Peptides were resuspended in 31 µL of 0.1% formic acid in water, protein quantified by NanoDrop and the concentration adjusted to 0.1 µg/µL and 5 µL was injected. Chromatography was performed at a continuous flow rate of 250 nL/min, in a 15 cm analytical column with 75 µm internal diameter, containing 3 µm diameter C18 particles. The chromatography mobile phases included phase A of 0.1% formic acid and phase B of 0.1% formic acid and 95% acetonitrile. The sample was then separated using a gradient from 5 to 25% mobile phase B in 80 min and from 25 to 40% of mobile phase B in 10 min. The precursor ion search was conducted with 100–1,700 m/z at 120000 resolution. Automatic gain control (AGC) was standard and maximum injection time of 50 ms. Loads 1 and greater than 5 were excluded. Dynamic exclusion time of 60 s was used. Samples were run in duplicate.

2.11 Analysis of peptide characteristics and identification of bioactive potential

The peptides from the AP and BL chamber were identified with PatternLab for Proteomics (Carvalho et al., 2016) and NOVOR software (MA, 2015)

(available in https://novor.cloud/), using predefined definitions and the Helianthus annuus and Bos Tauros database as reference. Once identified, they were screened for bioactive potential using the food peptide databases, BIOPEP-UWM (https://biochemia.uwm.edu.pl/ en/biopep-uwm-2/) and MBPDB 2.0 (https://mbpdb.nws.oregonstate.edu/). Biological functionality was related to structural aspects of the peptide such as origin (UniProt), hydrophobicity (GRAVY- http://www.gravy-calculator.de), isoelectric point (Bioinformatics, http://isoelectric.org/), size and prevalence of the peptide (PatternLab).

2.12 Statistical analysis

The data generated were statistically analyzed through analysis of variance (ANOVA). The mean ± standard deviation (SD) was used to represent the data of the experiments of the physicochemical composition (total phenolic compounds, degree of hydrolysis and determination of proteins). One-way ANOVA followed by Tukey's post hoc test and Duncan's test for multiple comparison was performed in the molecular weight distribution (FPLC) and total amino acid experiments. One-way ANOVA was performed in the *in vitro* experiments (cytotoxicity and Lucifer Yellow). In all analyses, differences were considered significant when $p < 0.05$. Data analysis was performed using the Statistical Package for Social Science, SPSS (version 16.0, Systat Software, Inc., Chicago IL).

3 RESULTS AND DISCUSSION

3.1 Digestion of sunflower and whey protein isolates and chemical characterization of peptides

Digestion of sunflower protein isolate (SPI) and whey protein isolate (WPI) was performed using equivalent amounts of protein, respectively 89.4±1.9% and 84.6±0.6%, (Table 1). SPI was purified from defatted sunflower meal (DSM), whereas WPI was obtained from a commercial supplier. The protein content increased from 48.0% in the DSM to 89.4% in the SPI, demonstrating the effectiveness of the DSM protein isolation procedure. SPI is rich in phenolic content which may reduce the digestibility and solubility of sunflower protein (Pérez-Gregorio et al., 2020). Therefore, to compare the absorption of peptides

with animal protein (WPI), the DSM had its phenolic content levels reduced, to avoid interference in digestion. SPI showed a reduction of over 80% in sunflower phenolic compound (from 256 to 38 AG mg/100g).

Both whey protein digestion (WPD) and sunflower protein digestion (SPD) exhibited comparable degrees of hydrolysis (DH) during the simulated digestion process, reaching 60.4% and 62.1%, respectively (Table 1). The degree of protein hydrolysis achieved by simulated gastrointestinal digestion can vary greatly depending on the protocol used, but also on the food matrix (origin and complexity of the sample) and sample processing (protein denaturation). Generally, animal-derived proteins exhibit higher digestibility than plant-based sources; specifically, whey protein isolate typically demonstrates superior hydrolysis compared to various plant proteins under the same protocol, such as sorghum, wheat bran cereals and black beans (Sousa et al., 2020). No single study reported the degree of hydrolysis (DH%) for both sunflower protein isolate and whey protein isolate under the same simulated gastrointestinal (SGID) protocol.

The comparable digestion values observed in this study may be attributed to the reduced phenolic content in SPD, as these compounds are known to hinder digestion. Reducing non-protein components may increase protein solubility and accessibility to gastrointestinal enzymes (Kaur & Ghoshal, 2022). Consistently, both soy and lentil protein isolates exhibit significantly higher digestibility than their respective flours (Barbana & Boye, 2013; Schimbator et al., 2020).

Table 1: Protein results, degree of hydrolysis and phenolic compound content.

Samples	Protein (%) ± SE	Degree of hydrolysis (%) ± SD	Phenolic reduction (Ag mg/100g)
DSM	48.0± 0.4^{a}		256 ± 0.4^{a}
SPI	89.4 ± 1.9^{b}	62.1 ± 3.9^{a}	38 ± 0.14^{b}
SPD	65.7 ± 0.4^{a,b}		Nd
WPI	84.6 ± 0.6^{b}		Nd
WPD	62.0 ± 0.2^{a,b}	60.4 ± 1.4^{a}	Nd

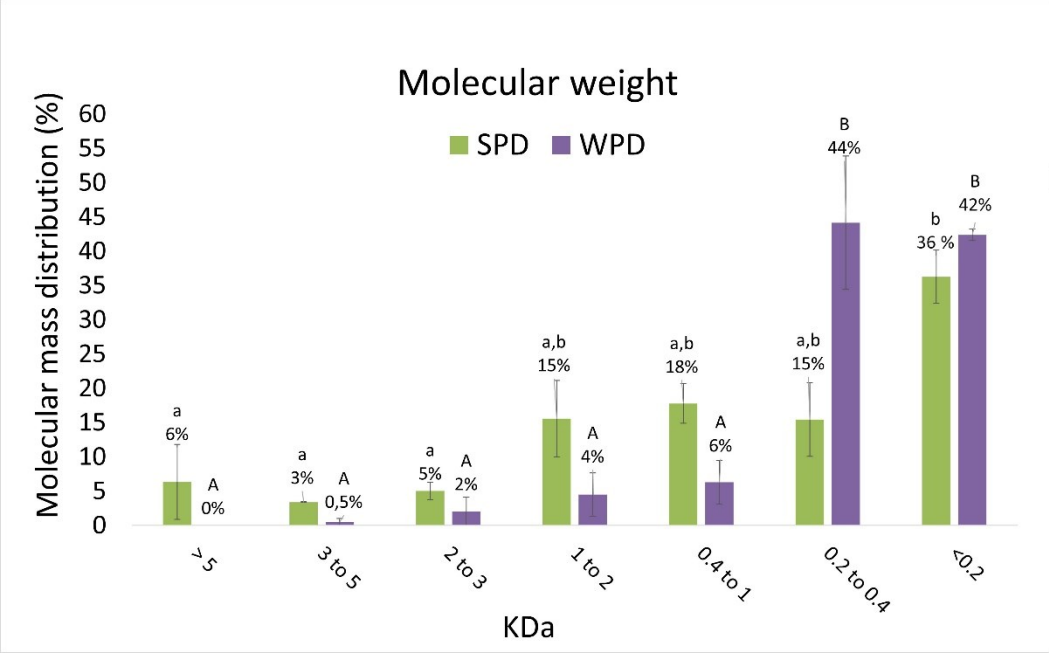
The abbreviation includes Defatted Sunflower Flour (DSM), Sunflower Protein Isolate (SPI), Sunflower Protein Digestion (SPD), Whey Protein Isolate (WPI), Whey Protein Digestion (WPD), and Indeterminate (n.d.). Values expressed as mean ± standard error (SE). Statistical analysis

was performed by Kruskal-Wallis test or T test with a significance level of $p < 0.05$. Different lowercase letters indicate statistical difference.

3.2 Molecular weight distribution of whey and sunflower protein digestion

The molecular weight distribution of the peptides following simulated gastrointestinal digestion (Figure 1) revealed some differences between whey (WPD) and sunflower (SPD). Both proteins were extensively degraded into very small fragments, primarily free amino acids (< 0.2 kDa), a fraction that was larger for WPD (44.8%) than for SPD (36.3%). The remaining peptides also showed distinct profiles.

A large proportion of WPD (44.2%) was concentrated in the 0.2−0.4 kDa range, indicating a highly efficient production of very small peptides (2 to 5 amino acids long). In contrast, the SPD peptides exhibited a broader distribution across three larger size categories, with similar quantities observed in the 1.0−0.4 kDa (17.8%), 0.4−0.2 kDa (15.4%), and 2.0−1.0 kDa (15.6%) ranges. This difference confirms that, under the simulated conditions, whey protein was better hydrolyzed into amino acids and ultra-small peptides than sunflower protein.



3.3 Cellular Caco-2 integrity and cytotoxicity of hydrolysates

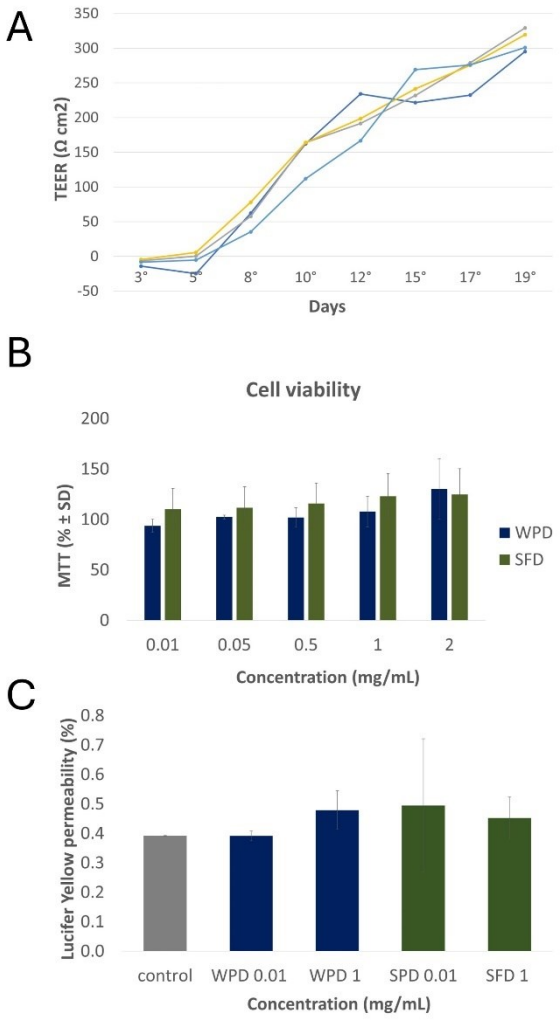
The integrity of the monolayers was assessed by TEER measurements (Fig. 3A). Initially, Caco-2 cells were cultured in 12-well transwell inserts for 19

days until they showed enterocyte phenotype and TEER values >250 Ω, which are indicative of good integrity (Liang et al., 2022).

Hydrolyzed products in their concentrated form can be cytotoxic to cells (Corrochano et al., 2019). For this reason, samples were desalinated and different hydrolyzed concentrations from *in vitro* simulated gastrointestinal digestion were investigated in human Caco-2 cell monolayers, prior to transportability studies. Previously, 6 concentrations were tested (0.01, 0.05, 0.1, 0.5, 1.0, and 2.0 mg/mL protein) by the MTT assay to ensure that the peptide concentrations used in this study did not significantly affect cell viability. As shown in Fig.3B, the samples had no significant cytotoxic effect on the concentrations tested for Caco-2.

The concentration of 1 mg/mL was selected based on concentrations used in previous Caco-2 protein transport studies and to ensure sufficient sensitivity for subsequent proteomic detection and analysis (Corrochano et al., 2019; Liang et al., 2022). Furthermore, 1 mg/mL concentration (corresponding to a final density of 250 μg/cm^2) simulates high-physiological protein exposure. Based on the daily reference value of 50 g of protein per day and an average small intestinal surface area of 200 m^2, the baseline physiological protein density is approximately 25 μg/cm^2. However, *in vitro* models lack the complex microclimate and three-dimensional surface area (villi and microvilli) present *in vivo*. Consequently, literature suggests a compensatory increase (5 to 10-fold) to mimic actual cellular exposure, ensuring the model remains both physiologically relevant and robust for cytotoxicity and transport assessments.

To guarantee the integrity of the cell monolayer after treatment with SPD and WPD, the transportability of lucifer yellow fluorescent dye (LY) was tested in the same set of cells. In Fig.3C, no statistical difference in the permeability of the LY dye is shown compared to the control. Therefore, during the transport assay, the integrity of the Caco-2 monolayer cells was preserved.



3.4 Total amino acid transport

Table 2 displays the total amino acid quantification in the apical (AP) and basolateral (BL) compartments of Caco-2 cells exposed to WPD and SPD during 2 h. Is is important to consider that the overall amount of total amino acids recovered in the basolateral chamber is the result not only of free amino acid but also peptide transport across the cell monolayer. As expected, the total amino acids measured in the AP chamber (36.0 mg/100 g for WPD and 39.03 mg/100 g for SPD) are significantly higher than in the BL chamber (3.37 mg/100 g for WPD and 2.68 mg/100 g for SPD), indicating that a considerable portion of amino acids and peptides are not transported. The proportion of total amino acid content presented in the BL compared to the AP chamber was greater for WPD (9.3%) than for SPD (6.8%).

The rate and efficiency of amino acid transport are influenced by the concentration of both the specific amino acid and other amino acids present. This

occurs through mechanisms like transporter saturation, competition for common transport systems, and trans-stimulation (Bröer & Fairweather, 2018).

Hydrophobic amino acids predominate in both the AP and BL compartments for both samples, with no variation in proportion after transport (Table 2, % of AA class column). In both samples, the basolateral (BL) increase in positive and aromatic amino acids, coupled with a decrease in neutral amino acids, suggesting that transport was mediated by a cationic/neutral amino acid antiporter. In this system, intracellular neutral amino acids are exchanged to accelerate the uptake of cationic species (Bröer & Fairweather, 2018). Interestingly, sunflower protein is limited in Lys (positive), however the rate of absorption was higher compared to other amino acids in the sample.

The amino acid Asp and Glut were prevalent in both samples in apical and basolateral compartment. Interestingly, these amino acids, which are anionic amino acids present a Na^+ dependent common transport system, EAAT3, which can favor its absorption process (Bröer & Fairweather, 2018).

Overall, this analysis indicates that the amino acid/peptide profile between compartment (AP and BL) varies during gastrointestinal transport. In general, WPD presented greater rates of transport between AP and BL chamber than SPD, which could indicate better absorption rates of amino acids and peptides.

Table 2. Amino Acid Profiles in Apical and Basolateral Compartments after Treatment with Whey (WPD) and Sunflower Protein Digests (SPD).

Amino acids (AA)			WPD (mg/100g)					SPD (mg/100g)				
			AP	% of AA class	BL	% of AA class	Rate BL/AP (%)	AP	% of AA class	BL	% of AA class	Rate BL/AP (%)
Hydrophylic	Positively charged	Arg	0.155	**6.2**	0.113	**16.4**	73.2	0.540	**6.4**	0.015	**13.1**	2.7
		Lys	1.303		0.270		20.7	0.904		0.236		26.1
		His	0.776		0.167		21.5	1.046		0.102		9.7
	Negatively charged	Asp	3.835	**28.5**	0.215	**26.8**	5.6	4.504	**31.9**	0.162	**25.1**	3.6
		Glu	6.442		0.689		10.7	7.962		0.510		6.4
	Uncharged /neutral	Thr	2.847	**17.3**	0.144	**7.8**	5.0	1.097	**10.3**	0.165	**6.4**	15.0
		Ser	1.845		0.114		6.2	1.210		0.006		0.5
		Cys	0.154		ND		---	0.175		ND		---
	Aromatic	Tyr	0.059	**5.2**	0.011	**31.1**	19.1	0.188	**16.5**	0.016	**38.5**	8.3
Hydrophobic	Aliphatic	Gly	0.970	**46.3**	0.036	**45.7**	3.7	2.209	**46.5**	0.037	**49.6**	1.7
		Ala	1.645		0.159		9.7	1.093		0.145		13.3
		Val	1.928		0.076		3.9	2.383		0.053		2.2
		Leu	2.869		0.220		7.7	2.706		0.146		5.4
		Ile	3.050		0.068		2.2	2.628		0.056		2.1
		Met	ND		ND		---	ND		ND		---
		Pro	4.961		0.043		0.9	2.602		0.016		0.6
	Aromatic	Phe	1.275		0.935		73.4	4.543		0.875		19.3
Total			**36.02**		**3.37**		9.34	**39.03**		**2.68**		**6.87**

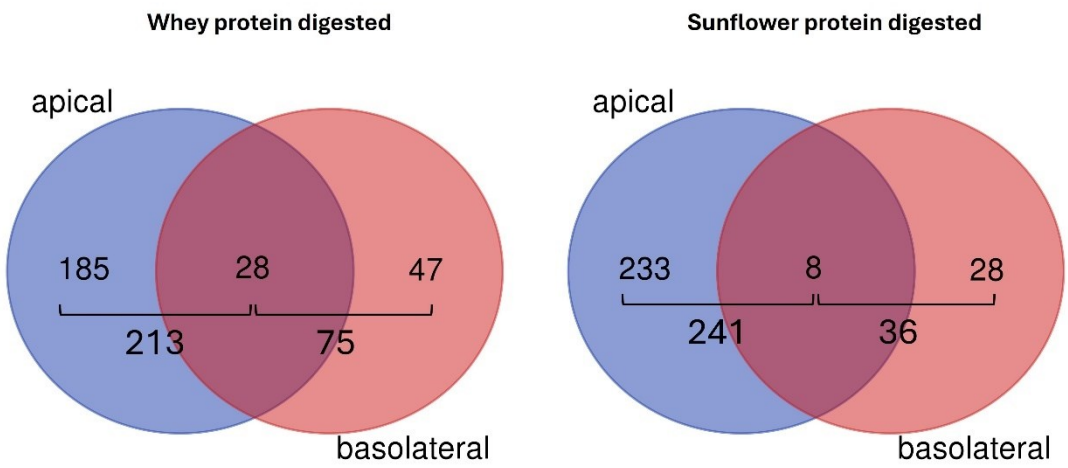
3.5 Transepithelial transport of peptides through Caco-2 cell monolayers.

The transportability of WPD and SPD peptides through the apical and basolateral chamber of the Caco-2 culture monolayer was tested after 2 h of incubation. Figure 3 shows the number of peptides found in the AP and BL chambers of WPD and SPD exposed cells. The identification of all peptides for each sample are shown in Suppl. Material Tables.

In the WPD sample, 75 out of 213 peptides crossed from the apical (AP) to the basolateral (BL) chamber, representing a transport efficiency of 35.2% in terms of sequence diversity. For the SPD sample, 36 out of 241 peptides were identified in the BL chamber, representing 14.9%. Lower peptide variability may represent a smaller quantity of peptides between chambers, suggesting that SPD samples had lower absorption rates compared to WPD.

Transport rates for peptide hydrolysates of animal origin show wide variability across studies utilizing the Caco-2 gastrointestinal barrier model. For instance, a 25% transport rate was reported for whey peptides at 0.5 mg/mL, which contrasts greatly with the 74% rate found by with milk peptides at a higher concentration (1 mg/mL) and higher time exposure (12h in peptide incubation) (Corrochano et al., 2019; Liang et al., 2022). Wang et al., (2019) observed a 60% transport rate for egg white hydrolysates, but at an even greater concentration of 5 mg/mL. The transport of myofibrillar protein hydrolysates was around 35 % at 0.5 mg/mL during 2h (Ye et al., 2022). Transport rates for hydrolysates derived from plant matrices were not found in the literature.

A key difference observed between the samples lies in the number of intact peptides transported. For the whey-exposed cells, 28 peptide sequences (9.8% of the total) were found identically in both the AP and BL compartments (Figure 4). This strongly suggests that these peptides resisted cleavage by brush border membrane peptidases and were transported intact across the Caco-2 cell monolayer. In contrast, the sunflower samples showed significantly lower transport, with only 8 (2.8%) identical peptides found between the compartments. These confirm that the source of the protein hydrolysate may impact its bioaccessibility and the structural integrity of peptides available for systemic absorption.



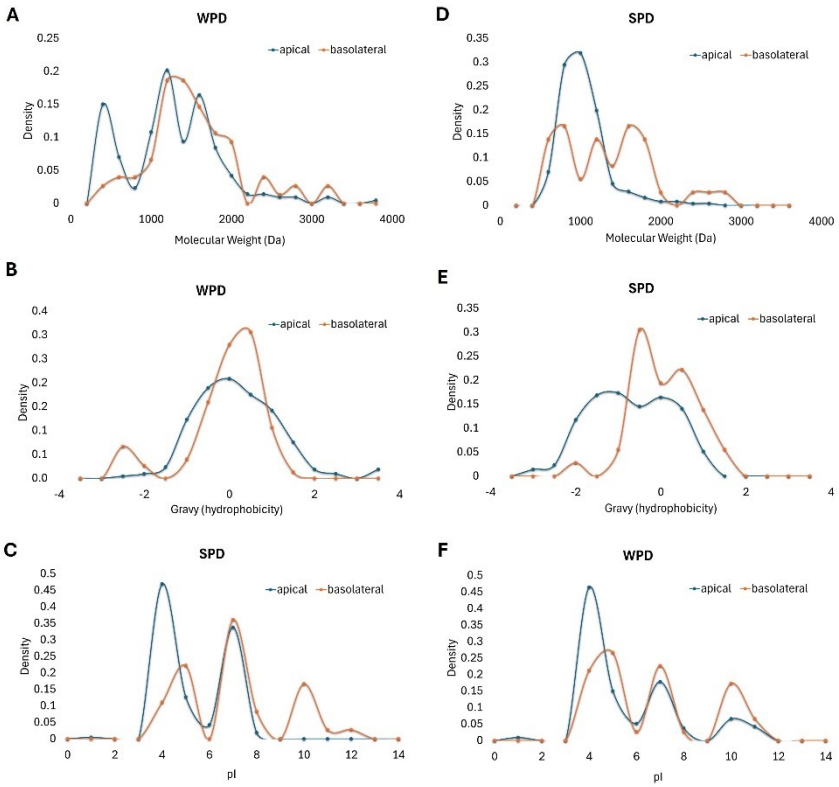
3.6 Physicochemical characteristics of transported peptides

Peptides identified in the apical (AP) and basolateral (BL) chambers were evaluated based on their physicochemical characteristics. Figure 4 illustrates peptide density in relation to molecular weight (MW), hydrophobicity, and isoelectric point for both WPD and SPD samples. Contrary to the FPLC analysis, LC/MS/MS data revealed that WPD peptides in the AP chamber exhibited greater MW variation than those in the SPD sample. However, in the basolateral chamber, WPD peptides occupied a narrower range (1000–2000 Da), while SPD peptides were distributed across a wider range (500–2000 Da) (Fig. 4A and 4D). This suggests that SPD transport is more variable regarding molecular size, potentially indicating that the two samples utilize distinct transport pathways. Furthermore, contrary to expectations, the mean molecular weight of peptides in the basolateral chamber was not lower than that of the apical chamber for either sample.

Peptide hydrophobicity was assessed using the Grand Average of Hydropathy (GRAVY) index, where positive values indicate hydrophobicity and negative values signify hydrophilicity. It is observed that in the BL compartment the hydrophobicity range becomes narrower for both samples and shifted to the right (in more positive range), indicating that the absorbed peptides were more hydrophobic compared to the AP compartment. For both samples, the peptides identified in the AP chamber were distributed in acidic pI ranges (4.0 and 7.0), while those identified in the BL chamber showed a distribution in higher pI ranges

(5.0, 7.0, and 10.0) and were therefore more basic. Previous studies showed that hydrophobic peptides from casein presented higher transport ratio (Wang and Li, 2018). Furthermore, hydrophobicity may contribute to better interaction with the phospholipid membrane, assisting in the transport of the peptide between compartments (Xie et al., 2015).

In general, the sequences recovered from the BL chamber were characterized by intermediate molecular weights, higher hidrophobicity, and a predominantly basic nature, resulting in a net positive charge. Factors such as size, electrical charge, and hydrophobicity directly influence the transport route a peptide uses and determine the efficiency with which these peptides reach the bloodstream. Small, hydrophilic peptides tend to pass through the paracellular route, via tight junctions, while small, hydrophobic peptides more easily cross the lipid bilayer by passive diffusion. Basic peptides with a high pI tend to avoid carrier-mediated transport routes such as PepT1 and the paracellular pathway, being preferentially transported by transcytosis, that is, through interaction with the plasma membrane and internalization into the cell by the formation of endocytic vesicles in an energy-dependent process. This type of transport is especially performed when peptides have longer chains (four or more amino acids) and are hydrophobic properties (Xu et al., 2019c). According to the physical-chemical characteristics of the identified peptides, transcytosis must be the most likely method to occurs in our sample, since they have intermediate size and are positively hydrophobic (Amigo & Hernández-Ledesma, 2020).



Another reason that may explain the difference in peptide transportability between the whey and sunflower hydrolysates may be the phenolic residual in SPD samples. Despite the reduction of phenolic compounds from sunflower samples, it is very difficult to achieve the complete removal, since the interaction between polyphenol and protein can occur in different ways, from non-covalent (H bonding, electrostatic interactions) or covalent bonds (Quan et al., 2019). Furthermore, the conjugation of proteins and nonpolar polyphenols could increase surface hydrophobicity modifying peptide structure and the internalization mechanism of the peptide. The irreversible interaction of protein-polyphenol conjugation occurs after oxidation, inducing the reaction of quinone of the polyphenol to the amino group of the amino acid. This reaction is also known to affect the bioavailability of amino acids (Cirkovic Velickovic & Stanic-Vucinic, 2018).

To assess the bioactive potential of the transported peptides, the sequences in the BL chamber of cells were cross-referenced with bioactivity databases. Table 3 shows the peptides transported from WPD with antimicrobial, antihypertensive (ACE inhibitor), antidiabetic (DPP-IV inhibitor), anticancer, and antioxidant activity. Meanwhile, peptides transported from SPD were found to

have bioactivities such as antithrombotic, antioxidant, antihypertensive, anxiolytic, and antidiabetic. Most of the peptides with correlated bioactivity were short peptides, indicating a greater chance of them being transported and absorbed. These findings demonstrate that while WPD and SPD yield different bioactivity profiles, both produce peptides capable of crossing the cellular barrier and present potential to exert systemic health benefits.

Table 3. Identification and Homology of Basolateral Peptides from Whey and Sunflower Protein Digests to Known Bioactive Sequences

Sequence	Precursor protein	Function	Author/Reference
		WPD 1.0 mg/mL	
MMK	Kappa-casein	Antimicrobial	(Sedaghati et al., 2014)
VR	Serum albumin	ACE inhibitor	(Gómez-Ruiz et al., 2007)
	Serum albumin	DPP-IV inhibitor	(Nongonierma & FitzGerald, 2013)
YK	Alpha-S1-casein	Anticancer	(Otani & Suzuki, 2003)
YQK	Alpha-S2-casein	ACE inhibitor	(Contreras et al., 2013)
	Alpha-S2-casein	Antioxidant	(Contreras et al., 2013)
YVPR	Alpha-S2-casein	Antioxidant	(Tonolo et al., 2019)
		SPD 1.0 mg/mL	
CLCR	Muscle proteins	Antithrombotic	(Nasri et al., 2012)
DAHK	Rice albumin	Antioxidant	(Wei et al., 2007)
LDLQR	Defatted wheat germ powder	Antidiabetic	(Liu et al., 2021)
LLAPPER	Autolyzed from squid viscera	Antioxidant	(Song et al., 2016)
MEMK	Hydrolysates of marine bivalves	Antioxidant	(Liu et al., 2015)
PLPR	Rice albumin	Contraction of the ileum	(Takahashi et al., 1996)
YLLLK	Palm kernel cake hydrolysates	Antioxidant	(Zarei et al., 2014)
YLPR	Ovalbumin	Anxiolytic	(Oda et al., 2012)
YLVR	Hazelnut protein	Anti-hypertensive	(Liu et al., 2018)

4 CONCLUSION

The results indicate that the whey protein (WPD) and sunflower protein (SPD) proteins were efficiently breakdown during simulated gastrointestinal digestion and that most of their proteins were degraded into free amino acids, with sizes less than 0.2 kDa. However, a big part of whey remained as low molecular weight peptides (0.2 - 0.4 kDa), while sunflower presented a wider distribution (between 0.2 to 2 kDa). Although both sources generate bioactive peptides, WPD exhibits a significantly higher transportability rate and greater enzymatic resistance to brush-border enzymes compared to SPD. Furthermore, the identification of a specific physicochemical profile for transport—independent of carriers—provides valuable guidelines for the development of functional ingredients with high systemic bioavailability. Overall, this study helps to understand which serum and sunflower-derived peptides are bioavailable across the intestinal barrier, the absorption mechanisms involving peptides and the Caco-2 cell monolayer, as well as aid in the production of peptides with potential for use as functional food ingredients and formulations.

CRediT authorship contribution statement

Thalyne Mariane Santana de Faria: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Funding acquisition, Project administration.
Eduarda Spagnol Sacilotto: Investigation, Methodology
Lilian G. Venâncio Carvalho Almeida: Investigation, Methodology
Daniele Cristina Napoli: Investigation, Methodology
Bruno C. Rossini: Methodology, Data curation, Software
Maria T. B. Pacheco: Investigation, Supervision
Fabiana Galland: Conceptualization, Data curation; Funding acquisition
Investigation, Project administration, Supervision, Writing – review and editing

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Declaration of Interest statement

The authors have no conflict of interest to declare. There are no known conflicts of interest associated with this publication and signifi cant financial support for this work that could have influenced its outcome.

Data availability

Data will be made available on request.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work the author(s) used Google Gemini in order to translate into English, improve writing fluency, and correct grammar. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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Figure 1. Molecular weight distribution (MW) of protein/peptide components in WPD and SPD samples. Each value is the mean (±standard error) of at least 2 experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey's test, with a significance level of $p < 0.05$. Lowercase letters indicate statistical difference between SPD and uppercase letters indicate statistical difference between WPD. Asterisc indicate statistical difference between WPD and SPD. WPD=whey protein digestion; SPD= sunflower protein digestion.

Figure 2. Cellular Caco-2 integrity and cytotoxicity of hydrolysates. (A) TEER values of the Caco-2 cell monolayer at different culture times. Different colors indicate four replicates. (B) Cell viability of different concentrations of WPD and SPD after 2 h of exposure. (C) Lucifer yellow (LY) solution (0.5 mM in HBSS) was added to the apical side after 2h of treatment with WPD and SPD, then HBSS was collected from the basal side and fluorescence intensity was measured. Each bar represents the mean and standard deviation of at least three determinations. Statistical analysis was performed by one-way ANOVA followed by Tukey's test, with a significance level of $p < 0.05$. WPD=whey protein digestion; SPD= digestion of sunflower protein.

Figure 3. Number of peptides found in the apical (blue circle) and basolateral (red circle) compartments of Caco-2 cell monolayers after 2h of treatment with WPD and SPD. The VennDiagran (https://bioinformatics.psb.ugent.be/webtools/Venn/) software was used as a tool to calculate the interactions. WPD=whey protein digestion; SPD=digestion of sunflower protein.

Figure 4. Physical-chemical characteristics of WPD and SPD peptides present in the apical and basolateral chamber. Density plots of peptides in each sample for the (A,D) molecular weight, (B,E) GRAVY score, (C,F) peptide isoelectric point.

5 CONCLUSÕES

Neste estudo, tratamos as monocamadas diferenciadas de uma linhagem celular de adenocarcinoma colorretal humano com proteínas de soro de leite e girassol submetidas à digestão gastrointestinal com o objetivo de identificar peptídeos que sobreviveram ao trânsito intestinal. Os resultados indicam que as proteínas WPD e SPD sofreram quebra durante a digestão gastrointestinal simulada e que a maioria de suas proteínas foi degradada em aminoácidos livres, com tamanhos inferiores a 0,2 kDa (44,8 e 36,3%, respectivamente). Os peptídeos foram gerados principalmente <20000 Da (76,3% para WPD e 69,6% para SPD), além disso, a hidrólise de proteínas séricas produziu peptídeos resistentes à absorção em células Caco-2.

Embora nenhum peptídeo bioativo tenha sido identificado a partir de digeridos de girassol, não se pode concluir que essa matriz não produziu peptídeos com propriedades biológicas, pois muitos peptídeos que atravessaram a monocamada podem ter bioatividades, mas ainda não foram testados quanto à função. Portanto, mais estudos devem ser realizados para examinar a capacidade das sequências de peptídeos bioativos de exercer bioatividades. No geral, este estudo ajuda a entender quais peptídeos derivados de soro e girassol são biodisponíveis através da barreira intestinal, os mecanismos de absorção envolvendo peptídeos e a monocamada de células Caco-2, além de auxiliar na produção de peptídeos com potencial para uso como ingredientes e formulações alimentares funcionais.

6 ANEXO - Supplementary material

The peptide sequences identified from the apical and basolateral chambers of the cells incubated with milk serum and sunflower proteins are presented.

Table S1. WPD 1.0 mg/mL apical compartment.

Sequence	Origen	Molecular Weight	N° of AA	Hydrophobicity	Isoelectric Point
AAGGPGAPADPGRPT	Polymeric immunoglobulin receptor	1290.6	15	-0.633	6.34
AAVEEGIVLGGGCALLR	60 kDa heat shock protein. mitochondrial	1683.9	17	1.124	4.26
AEDPFIAIH	Alpha-amylase	1011.5	9	0.400	4.18
AFVHWYVGEGMEEGEFSEAR	Tubulin alpha chain	2329.0	20	-0.495	4.02
AGFNAGA	Kelch Repeat and BTB Domain Containing	606.3	7	0.557	6.10
AILVDLEPGTMDSVR	Tubulin beta-2B chain	1614.8	15	0.380	3.88
ALPFWNEEIVPQIK	Phosphoglycerate mutase (dependent on 2.3-diphosphoglycerate)	1682.9	14	-0.029	4.26
ALPM	Beta-Lactoglobulin	430.6	4	1.475	3.8
ALPP	Beta-casein	396.5	4	0.600	3.9
ALQPNFEEDK	Prostaglandina-H2 D-isomerase	1189.6	10	-1.460	3.93
ALVFVDNH	Alpha-amylase	913.5	8	0.825	5.29
ALVFVDNHDNQ	Alpha-amylase	1270.6	11	-0.355	4.11
AVFVDLEPTVIDEIR	Tubulin alpha-4A chain	1714.9	15	0.613	3.74
AVFVDLEPTVIDEVR	Tubulin alpha chain	1700.9	15	0.593	3.74
AVVHGIVMGIPVPFPIPE	Intracellular cholesterol transporter NPC 2	1887.0	18	1.272	5.36
AVVRP	Kappa-Casein	540.7	5	0.820	10.6

CCTESLVNR	Albumin	1137.5	9	0.000	6.14
CDVISGDK	Alpha-amylase	1495.7	8	-0.113	4.11
CTEDYLSLILNR	Albumin	835.4	12	0.050	4.18
DAAGGPGAPADPGRPT	Polymeric immunoglobulin receptor	1405.7	16	-0.813	4.11
DAAGGPGAPADPGRPTG	Polymeric immunoglobulin receptor	1462.7	17	-0.788	4.11
DAQSAPLR	Beta-Lactoglobulin	856.9	8	-0.813	6.3
DATEEDFTSH	Secreted phosphoprotein 1	1150.4	10	-1.480	3.74
DATNVGDEGGFAPNILENK	Phosphopyruvate hydrates	1959.9	19	-0.684	3.74
DDDLTDDI	Alfa-lactalbumin	920.4	8	-1.238	3.74
DDDLTDDIM	Alpha-lactAlbumin	1067.4	9	-0.889	3.74
DDSPDLPK	Albumin	885.4	8	-1.825	3.83
DERF	Kappa-Casein	565.6	4	-2.175	3.9
DIDGIWEGTRD	Triacylglycerol lipase	1275.6	11	-1.082	3.70
DLLVAEHPQ	Carboxypeptidase A1	1020.5	9	-0.189	4.18
DLSKEPSISR	Glycosylation-dependent cell adhesion molecule 1	1130.6	10	-1.110	6.49
DLLTGIPPAP	Endoplasmic reticulum chaperone BiP	879.5	9	0.078	3.75
DLTGIPPAPR	Endoplasmic reticulum chaperone BiP	1035.6	10	-0.380	6.34
DLYANTVLSGGTTMYPGIADR	Beta actin	2214.1	21	-0.071	4.11
DNIQGITKPAIR	Histona H4	1324.7	12	-0.525	9.70
DQWL	Alfa-lactoalbumin	560.6	4	-1.025	0.7
DSTLIMQLLR	14-3-3 Zeta/Delta Protein	1188.7	10	0.480	6.34
DTDDVPVPA	Nucleobindine-1	927.4	9	-0.467	3.34
DVNAAIATIK	Tubulin alpha chain	1014.6	10	0.700	6.34
EAFSLFDKDGDGTITTK	Calmodulin 1	1843.9	17	-0.576	4.06
EDAANNYAR	Tubulin alpha chain	1022.4	9	-1.600	4.18

EELKPTPE	Lipocalin/cytosolic fatty acid-binding domain containing protein	941.5	8	-1.813	3.98
EITALAPSTMK	Actina beta	1160.6	11	0.236	6.41
EL	Alpha-S1-casein	260.3	2	0.150	0.9
ELASQPDVDGFLVGGASLKPEFVDIINAK	Actina beta	3028.6	29	0.197	3.92
ELQQGLQPV	Chymotrypsin	1010.5	9	-0.467	3.85
EMPFPK	Beta-casein	747.9	6	-0.983	6.9
ESGPSLVKP	Protein containing Ig-like domain	912.5	9	-0.511	6.41
ESGPSLVKPSQ	Protein containing Ig-like domain	1127.6	11	-0.809	6.41
ESGPSLVKPSQT	Protein containing Ig-like domain	1228.6	12	-0.800	6.41
ESPPEINT	Casein	885.4	8	-1.338	3.61
EVDDEALEKFD	Lipocalin/cytosolic fatty acid-binding domain containing protein	1308.6	11	-1.118	3.53
EVDDEALEKFDK	Lipocalin/cytosolic fatty acid-binding domain containing protein	1436.7	12	-1.350	3.82
EVDEQMLNVQNK	Tubulin beta-2B chain	1445.7	12	-1.192	3.93
FDGALNVDLTEFQTNLVPYPR	Tubulin alpha chain	2408.2	21	-0.219	3.88
FFVAP	Alpha-S1-casein	579.7	5	2.000	3.6
FHPDTDDVPVPAPA	Nucleobindine-1	1476.7	14	-0.429	3.83
FL	Lactotransferrin	278.4	2	3.300	3.4
FP	Serum Albumin	262.3	2	0.600	3.6
FQNALIVR	Albumin	959.6	8	0.700	10.55
GAW	Alpha-S1-casein	332.4	3	0.167	3.6
GFL	Beta-casein	335.4	3	2.067	3.6
GFQNALIVR	Albumin	1016.6	9	0.578	10.55

GGPGAPADPGRPT	Polymeric immunoglobulin receptor	1148.6	13	-1.008	6.34
GGSDDWAYDQGIK	Polymeric immunoglobulin receptor	1410.6	13	-1.215	3.83
GHLDFFPNGGK	Polymeric immunoglobulin receptor	1187.6	11	-0.682	7.55
GHYTEGAELVDSVLDVVR	Tubulin beta-2B chain	1958.0	18	0.050	4.10
GIPVPFPIPE	Intracellular cholesterol transporter NPC 2	1064.6	10	0.570	3.85
GLTGPIGPPGPAGANGEK	Collagen type II alpha chain 1	1589.8	18	-0.472	6.41
GNNISSGTVLSDYVGSGPPK	Phosphatidylethanolamine-binding protein 1	1948.9	20	-0.385	6.33
GVVDSEDLPLNISR	HSP 90-alpha Heat Shock Protein	1512.8	14	-0.114	3.88
GYSFTTTAER	Beta actin	1131.5	10	-0.800	6.40
HLDFFPNGGK	Triacylglycerol lipase	1130.6	10	-0.710	7.55
HLPLP	Beta-casein	575.7	5	0.240	7.6
HLVDEPQNLIK	Albumin	1304.7	11	-0.582	5.45
HMWPGDIK	Alpha-amylase	982.5	8	-0.888	7.55
HQGVMVGMGQK	Beta actin	1170.6	11	-0.282	9.70
IESPPEIN	Kappa-Casein	897.4	8	-0.688	3.61
IGGIGTVPVGR	Elongation factor 1-alpha 2	1024.6	11	0.818	10.55
IIPGFMCQGGDFTR	Peptidil-prolil cis-trans isomerase A	1597.7	14	0.286	6.16
ILNKPEDE	Glycosylation-dependent cell adhesion molecule 1	956.5	8	-1.400	3.93
IPSHAVVAR	Carbonic anhydrase inhibitor	948.6	9	0.711	10.55
ISEQFTAMFR	Tubulin beta-2B chain	1228.6	10	0.080	6.41
ISGLIYEETR	Histona H4	1179.6	10	-0.190	4.26
ISLPLPNFSSLNLR	Vimentin	1569.9	14	0.386	10.55
IWHHTFYNELR	Beta actin	1514.7	11	-0.882	7.71
IYVDAVINH	Alpha-amylase	1042.5	9	0.856	5.29

IYVDAVINHM	Alpha-amylase	1173.6	10	0.960	5.29
KVESLQEEIAFLK	Vimentin	1532.8	13	-0.131	4.48
KVPQVSTPTLVEVSR	Albumin	1638.9	15	-0.067	9.70
KYP	Beta-casein	406.5	3	-2.267	9.6
LDDDLTDDI	Alpha-lactalbumin	1033.4	9	-0.678	3.14
LDDDLTDDIM	Alpha-lactalbumin	1180.5	10	-0.420	3.14
LDLALEKD	Alpha-amylase	915.5	8	-0.150	3.88
LDLALEKDYV	Alpha-amylase	1177.6	10	0.170	3.88
LF	Beta-Lactoglobulin	278.4	2	3.300	3.7
LFPPSTEELNGNK	Protein containing Ig-like domain	1444.7	13	-0.969	4.26
LGEYGFQNALIVR	Albumin	1478.8	13	0.292	6.40
LHFFMPGFAPLTSR	Tubulin beta-2B chain	1635.8	14	0.493	10.55
LHVDPENFR	Hemoglobina. beta	1125.6	9	-1.000	5.45
LIGQIVSSITASLR	Tubulin alpha chain	1456.9	14	1.114	10.55
LISWYDNEFGYSNR	Glyceraldehyde-3-phosphate dehydrogenase	1762.8	14	-0.921	4.18
LL	Beta-Lactoglobulin	244.3	2	3.800	3.6
LLF	Beta-Lactoglobulin	391.5	3	3.467	3.7
LLQDSVDFSLADAINTEFK	Vimentin	2125.1	19	0.111	3.70
LNVPGEIVESL	Beta-casein	1168.6	11	0.655	3.61
LP	Serum Albumin	228.3	2	1.100	3.8
LPL	Beta-casein	341.5	3	2.000	3.6
LPLP	Beta-casein	438.6	4	1.100	3.8
LPP	Beta-casein	325.4	3	0.200	3.8
LPQ	Beta-casein	356.4	3	-0.433	3.7
LQP	Beta-casein	356.4	3	-0.433	3.8
LTTPTYGDLNHLVSATMSGVTTCLR	Tubulin beta-2B chain	2707.3	25	0.224	7.36
LVQDVANNTNEEAGDGTTTATVLAR	60 kDa heat shock protein. mitochondrial	2559.2	25	-0.376	3.74
LVYP	Beta-casein	490.6	4	1.275	3.8
LVYPFPGPI	Beta-casein	1001.6	9	0.978	6.09

LVYPFPGPIP	Beta-casein	1098.6	10	0.720	6.09
LVYPFPGPIPN	Beta-casein	1212.7	11	0.336	6.09
LW	Alpha-S1-casein	317.4	2	1.450	3.6
LY	Lactotransferrin	294.4	2	1.250	3.6
LYTLVLTDPDAPSR	Proteína de ligação à fosfatidiletanolamina 1	1559.8	14	-0.057	4.11
MKP	Alfa-S2-Casein	374.5	3	-1.200	9.9
MMK	Kappa-Casein	408.6	3	-0.033	9.9
NLDIERPTYTNLNR	Tubulin alpha chain	1717.9	14	-1.336	6.49
NSLP	Beta-casein	429.5	4	-0.525	3.4
PFP	Beta-casein	359.4	3	-0.133	4.3
PFPGPIPN	Beta-casein	837.4	8	-0.375	6.10
PGASGLKGEPGAV	Colágeno tipo XXI cadeia alfa 1	1138.6	13	-0.108	6.41
PLP	Beta-casein	325.4	3	0.200	4.3
PVVVPPFLQP	Beta-casein	1091.6	10	0.930	6.10
PVVVPPFLQPE	Beta-casein	1220.7	11	0.527	3.85
PVVVPPFLQPEV	Beta-casein	1320.7	12	0.833	3.85
PVVVPPFLQPEVM	Beta-casein	1466.8	13	0.915	3.85
PVVVPPFLQPEVMG	Beta-casein	1523.8	14	0.821	3.85
QLFHPEQLITGK	Tubulin alpha chain	1409.8	12	-0.450	7.55
RHPEYAVSVLLR	Albumin	1438.8	12	-0.133	9.35
RHPYFYAPELLYYANKYNGVFQEC CQAEDK	Albumin	3772.7	30	-0.860	5.61
RNVVDGQPF	Alpha-amylase	1030.5	9	-0.644	6.34
RY	Alpha-S1-casein	337.4	2	-2.900	9.6
SDDWAYDQGIK	Carboxipeptidase B	1296.6	11	-1.364	3.83
SIQFVDWCPTGFK	Tubulin alpha-4A chain	1583.7	13	0.115	6.16
SISNSAEDPF	Alpha-amylase	1065.5	10	-0.540	3.55
SISNSAEDPFIAIH	Alpha-amylase	1499.7	14	0.157	4.18
SLGLTDDHI	Lipocalina 2	969.5	9	-0.002	4.11
SLHTLFGDELCK	Albumin	1418.7	12	0.058	5.45
SLPQ	Beta-casein	443.5	4	-0.525	3.4

SLVYPFPGPI	Beta-casein	1088.6	10	0.800	6.09
SLVYPFPGPIP	Beta-casein	1185.6	11	0.582	6.09
SNEDLSKEPSISR	Glycosylation-dependent cell adhesion molecule 1	1460.7	13	-1.454	4.43
SNSAEDPFIA	Alpha-amylase	1049.5	10	-0.280	3.55
SNVQSPDATEED	Secreted phosphoprotein 1	1290.5	12	-1.575	3.29
SNVQSPDATEEDFTSH	Secreted phosphoprotein 1	1762.7	16	-1.300	3.74
SPPSVTLFPPSTEEL	Protein containing Ig-like domain	1599.8	15	-0.173	3.61
SPPSVTLFPPSTEELNGNK	Protein containing Ig-like domain	2013.0	19	-0.732	4.26
SQIVDIDGIWEGTRD	Triacylglycerol lipase	1702.8	15	-0.500	3.70
SVTEQGAELSNEER	14-3-3 Zeta/Delta Protein	1547.7	14	-1.314	3.82
SYELPDGQVITIGNER	Beta actin	1789.9	16	-0.638	3.93
SYPGQIT	Tripsina aniônica	764.4	7	-0.543	6.09
TF	Beta-casein	266.3	2	1.050	3.4
TGAIVDVPVGEELLGR	ATP sintase subunidade alfa	1623.9	16	0.500	3.93
TIGGGDDSFNTFFSETGAGK	Tubulin alpha chain	2006.9	20	-0.445	3.88
TLNDELEIIEGMK	60 kDa heat shock protein. mitochondrial	1503.7	13	-0.308	3.78
TPEVDDEA	Lipocalin/cytosolic fatty acid-binding domain containing protein	874.4	8	-1.288	3.29
TPEVDDEALEKFDK	Lipocalin/cytosolic fatty acid-binding domain containing protein	1634.8	14	-1.321	3.82
TTGIVMDSGDGVTHTVPIYEGYALPH	Beta actin	2729.3	26	0.046	4.36
TTGIVMDSGDGVTHTVPIYEGYALPHAILR	Beta actin	3182.6	30	0.227	5.36
TVTAMDVVYALK	Histone H4	1309.7	12	0.983	6.33
TYFPHFDLSHGSAQVK	Proteína contendo domínio de globina	1832.9	16	-0.469	7.70

VAPEEHPVLLTEAPLNPK	Beta actin	1953.1	18	-0.267	4.47
VAPFPE	Alpha-S1-casein	658.8	6	0.350	3.9
VAV	Kappa-Casein	287.4	3	3.400	3.6
VAVLGASGGIGQPLSLLLK	Malato desidrogenase mitocondrial	1792.1	19	1.226	9.70
VDIDGIWEGTRD	Triacylglycerol lipase	1374.6	12	-0.642	3.70
VEELKPTPE	Lipocalin/cytosolic fatty acid-binding domain containing protein	1040.5	9	-1.144	3.98
VEELKPTPEGDLE	Lipocalin/cytosolic fatty acid-binding domain containing protein	1454.7	13	-1.069	3.67
VEWELTDDKN	Intracellular cholesterol transporter NPC 2	1247.6	10	-1.500	3.74
VGGHAAEYGAEALER	60 kDa heat shock protein. mitochondrial	1528.7	15	-0.367	4.47
VGGTSDVEVNEK	60 kDa heat shock protein. mitochondrial	1232.6	12	-0.633	3.93
VGLQVVAVK	60 kDa heat shock protein. mitochondrial	911.6	9	1.622	9.70
VIESPPEINT	Kappa-Casein	1097.6	10	-0.200	3.61
VIISAPSADAPMFVMGVNHEK	Glyceraldehyde-3-phosphate dehydrogenase	2212.1	21	0.514	5.45
VL	Lactotransferrin	230.3	2	4.000	3.6
VLQATVVAVGSGSK	10 kDa heat shock protein. mitochondrial	1314.8	14	0.979	9.70
VNQIGSVTESLQACK	Phosphopyruvate hydrates	1632.8	15	0.027	6.23
VPP	Beta-casein	311.4	3	0.333	3.8
VPQVSTPTLVEVSR	Albumin	1510.8	14	0.207	6.41
VR	Serum Albumin	273.3	2	-0.150	10.8
VTIAQGGVLPNIQAVLLPK	Histone H2A	1930.2	19	0.921	9.70
VVGGEDARPN	Member of the elastase family similar to Chymotrypsin 2A	1012.5	10	-0.720	4.18

VVGGEDARPNSWPW	Member of the elastase family similar to Chymotrypsin 2A	1568.7	14	-0.814	4.18
VVLDDKDYFLFR	10 kDa heat shock protein. mitochondrial	1528.8	12	0.117	4.31
VVPP	Beta-casein	410.5	4	1.300	3.8
VVVEWELTDDK	Intracellular cholesterol transporter NPC 2	1331.7	11	-0.282	3.74
VVVEWELTDDKNQ	Intracellular cholesterol transporter NPC 2	1573.8	13	-0.777	3.74
VVVPPFLQPEV	Beta-casein	1222.7	11	1.055	3.85
VY	Beta-casein	280.3	2	1.450	3.6
VYP	Beta-casein	377.4	3	0.433	3.7
VYPFPGPI	Beta-casein	888.5	8	0.625	6.09
VYPFPGPIP	Beta-casein	985.5	9	0.378	6.09
VYPFPGPIPN	Beta-casein	1099.6	10	-0.010	6.09
VYVEELKPTPEGDLE	Lipocalin/cytosolic fatty acid-binding domain containing protein	1716.8	15	-0.733	3.67
WL	Alfa-lactoalbumin	317.4	2	1.450	3.5
WV	Alfa-lactoalbumin	303.4	2	1.650	3.5
YFPTQALNFAFK	ADP/ATP translocase	1445.7	12	0.108	9.30
YL	Alpha-S1-casein	294.4	2	1.250	3.3
YP	Lactotransferrin	278.3	2	-1.450	3.5
YPFP	Beta-casein	522.6	4	-0.425	3.5
YPFPGP	Beta-casein	676.8	6	-0.617	3.5
YPIEHGIITNWDDMEK	Actin. alpha 1 heart muscle	1959.9	16	-0.881	4.10
YVDAVINH	Alpha-amylase	929.5	8	0.400	5.29
YVEELKPTPEGDLE	Lipocalin/cytosolic fatty acid-binding domain containing protein	1617.8	14	-1.086	3.67
YVL	Kappa-Casein	393.5	3	2.233	3.3
YVPR	Kappa-Casein	533.6	4	-0.800	9.6

Table S2. WPD 1.0 mg/mL basolateral compartment

Sequence	Origen	Molecular Weight	N° of AA	Hydrophobicity	Isoelectric Point
ADLINNLGTIAK	HSP 90-alpha Heat Shock Protein	1241.7	12.00	0.392	6.34
AFVHWYVGEGMEEGEFSEAR	Actin. alpha 1 heart muscle	2329.0	20.00	-0.495	4.02
AGFAGDDAPR	Cytoplasmic Actin 1	975.4	10.00	-0.570	4.11
AGLQFPVGR	Histone H2A	943.5	9.00	0.244	10.55
ALPFWNEEIVPQIK	Phosphoglycerate mutase (dependent on 2.3-diphosphoglycerate)	1682.9	14.00	-0.029	4.26
AVFVDLEPTVIDEIR	Actin. alpha 1 heart muscle	1714.9	15.00	0.613	3.74
AVLVDLEPGTMDSVR	Tubulin beta-4B chain	1600.8	15.00	0.360	3.88
AVTEQGAELSNEER	14-3-3 Protein Teat	1531.7	14.00	-1.129	3.82
DLTDYLMK	Cytoplasmic Actin 1	997.5	8.00	-0.425	4.11
DLYANTVLSGGTTMYPGIADR	Cytoplasmic Actin 1	2214.1	21.00	-0.071	4.11
DQVANSAFVER	HSP 90-alpha Heat Shock Protein	1234.6	11.00	-0.409	4.18
DSTLIMQLLR	14-3-3 Zeta/Delta Protein	1188.7	10.00	0.480	6.34
DYDEGQDDRP	Fibrinogen beta chain	1208.5	10.00	-2.880	3.57
EDQTEYLEER	HSP 90-alpha Heat Shock Protein	1310.6	10.00	-2.370	3.67
EEQFNSTYR	Protein containing Ig-like domain	1172.5	9.00	-2.056	4.26
ELASQPDVDGFLVGGASLKPEFVDIINAK	Triosephosphate isomerase	3028.6	29.00	0.197	3.92
ELIDLVLDR	Tubulin alpha chain	1084.6	9.00	0.567	3.88
ELISNSSDALDK	Heat shock protein	1290.6	12.00	-0.533	3.88
EYQDLLNVK	Vimentin	1120.6	9.00	-0.822	4.18
FDGALNVDLTEFQTNLVPYPR	Actin. alpha 1 heart muscle	2408.2	21.00	-0.219	3.88
FPGQLNADLR	Beta-4B Tubulin Chain	1129.6	10.00	-0.480	6.34
GDLGIEIPAEK	Pyruvate kinase	1140.6	11.00	-0.200	3.93

GDYPLEAVR	Pyruvate kinase	1018.5	9.00	-0.556	4.18
GHYTEGAELVDSVLDVVR	Beta-4B Tubulin Chain	1958.0	18.00	0.050	4.1
GYISPYFINTSK	60 kDa heat shock protein. mitochondrial	1388.7	12.00	-0.208	9.15
HFSVEGQLEFR	HSP 90-alpha Heat Shock Protein	1347.7	11.00	-0.527	5.49
HQGVMVGMGQK	Cytoplasmic Actin 1	1170.6	11.00	-0.282	9.7
IHFPLATYAPVISAEK	Tubulin alpha chain	1756.0	16.00	0.538	7.54
IIAPPER	Cytoplasmic Actin 1	794.5	7.00	-0.057	6.41
ILLAELEQLK	Vimentin	1168.7	10.00	0.710	4.26
IMNTFSVVPSPK	Tubulin beta-4B chain	1318.7	12.00	0.392	9.7
INVYYNEATGGNYVPR	Tubulin beta chain	1828.9	16.00	-0.675	6.39
ISLPLPNFSSLNLR	Vimentin	1569.9	14.00	0.386	10.55
KEGGLGPLNIPLLADVTR	Peroxiredoxin-2	1862.1	18.00	0.094	6.49
KPLVIIAEDVDGEALSTLVLNR	60 kDa heat shock protein. mitochondrial	2364.3	22.00	0.500	4.11
KVESLQEEIAFLK	Vimentin	1532.8	13.00	-0.131	4.48
LAVNMVPFPR	Tubulin beta-4B chain	1142.6	10.00	0.750	10.55
LGEYGFQNALIVR	Albumin	1479.8	13.00	0.292	6.4
LHFFMPGFAPLTSR	Tubulin beta-4B chain	1619.8	14.00	0.493	10.55
LLEGEESR	Vimentin	931.5	8.00	-1.075	3.98
LLIEMEQR	Creatine kinase type B	1030.5	8.00	-0.125	4.26
LSVDYGK	Tubulin alpha chain	780.4	7.00	-0.271	6.33
LTTPTYGDLNHLVSATMSGVTTCLR	Tubulin beta-4B chain	2707.3	25.00	0.224	7.36
LVTDLTK	Albumin	788.5	7.00	0.429	6.34
MMK	Kappa-Casein	408.6	3.00	-0.033	9.88
QVTINDLPVGR	Peroxiredoxin-2	1210.7	11.00	-0.091	6.34
SGPFGQIFRPDNFVFGQSGAGNNWAK	Tubulin beta-4B chain	2797.3	26.00	-0.523	9.7
SIQFVDWCPTGFK	Tubulin alpha chain	1583.7	13.00	0.115	6.16
SLGGGTGSGMGTLLISK	Tubulin beta-4B chain	1534.8	17.00	0.453	9.7
SVTEQGAELSNEER	14-3-3 Zeta/Delta Protein	1547.7	14.00	-1.314	3.82
SYELPDGQVITIGNER	Cytoplasmic Actin 1	1789.9	16.00	-0.638	3.93

TDYDEGQDDRP	Fibrinogen beta chain	1309.5	11.00	-2.682	3.57
TIQFVDWCPTGFK	Tubulin alpha chain	1597.8	13.00	0.123	6.16
TITLEVEPSDTIENVK	Polyubiquitin-C	1786.9	16.00	-0.294	3.78
TTGIVMDSGDGVTHTVPIYEGYALPHAILR	Cytoplasmic Actin 1	3182.6	30.00	0.227	5.36
TVIIEQSWGSPK	60 kDa heat shock protein. mitochondrial	1343.7	12.00	-0.242	6.41
VAPEEHPVLLTEAPLNPK	Cytoplasmic Actin 1	1953.1	18.00	-0.267	4.47
VGINYQPPTVVPGGDLAK	Tubulin alpha chain	1825.0	18.00	0.017	6.33
VLQATVVAVGSGSK	Heat shock protein de 10 kDa. mitocondrial	1314.8	14.00	0.979	9.7
VLTPELYAELR	Creatine kinase type B	1302.7	11.00	0.209	4.26
VPQVSTPTLVEVSR	Albumin	1510.8	14.00	0.207	6.42
VR	Serum Albumin	273.3	2.00	-0.15	10.81
VTIAQGGVLPNIQAVLLPK	Histone H2A	1930.2	19.00	0.921	9.7
VVLAYEPVWAIGTGK	Triosephosphate isomerase	1601.9	15.00	0.787	6.4
VVLDDKDYFLFR	Heat shock protein de 10 kDa. mitocondria	1528.8	12.00	0.117	4.31
YDEGQDDRP	Fibrinogen beta chain	1093.4	9.00	-2.811	3.7
YFPTQALNFAFK	ADP/ATP translocase 2	1445.7	12.00	0.108	9.3
YK	Alpha-S1-casein	309.4	2.00	-2.6	9.49
YLAEVAAGDDKK	14-3-3 Zeta/Delta Protein	1278.6	12.00	-0.550	4.37
YLSEVASGDNK	14-3-3 beta/alpha protein	1181.6	11.00	-0.718	4.18
YLTVAAIFR	Tubulin beta chain	1052.6	9.00	1.378	9.35
YLYEIAR	Albumin	926.5	7.00	-0.071	6.4
YPIEHGIITNWDDMEK	Actin. aortic smooth muscle	1959.9	16.00	-0.881	4.1
YQK	Alfa-S2-Casein	437.5	3.00	-2.9	9.49
YVPR	Kappa-Casein	533.6	4.00	-0.8	9.57

Table S3. SPD 1.0 mg/mL apical compartment.

Sequence	Origen	Molecular Weight	N° of AA	Hydrophobicity	Isoelectric Point
AIFVDLEPTVIDEVR	Tubulin	1714.9	15	0.613	3.74
AIIVDDV	11-S Seed Storage Protein. Plant	743.4	7	1.743	3.49
ALDVAPPHGPL	Phythepsin	1085.6	11	0.318	5.29
ALEPIE	11-S Seed Storage Protein. Plant	670.4	6	0.250	3.61
ALGGTGTDHV	Dehydrin	926.4	10	0.050	5.29
ALVECEASPDL	Interactor Family C3H-WRC/GRF and Transcription Factor Regulator	1170.6	11	0.455	3.42
ANIASPA	11-S Seed Storage Protein. Plant	642.3	7	0.571	6.10
ANINDVSRPD	11-S Seed Storage Protein. Plant	1099.5	10	-1.040	4.11
APPHGPL	Phythepsin	687.4	7	-0.400	7.55
DAHK	Albuminde rice	469.5		-2.2	7.83
DAQEEEE	Tubulin	848.3	7	-2.743	3.26
DDMIQE	11-S Seed Storage Protein. Plant	749.3	6	-1.267	3.38
DDMIQEG	11-S Seed Storage Protein. Plant	806.3	7	-1.143	3.38
DDVNNPA	11-S Seed Storage Protein. Plant	743.3	7	-1.371	3.49
DDWVQEG	11-S Seed Storage Protein. Plant	847.3	7	-1.586	3.38
DDWVQEGQ	11-S Seed Storage Protein. Plant	975.4	8	-1.825	3.38
DENEWFP	RmlC-like termite domain superfamily. rmlC-like jelly roll protein	935.4	7	-1.957	3.42
DFVNPQA	11-S Seed Storage Protein. Plant	789.4	7	-0.471	3.75
DHHQ	Albuminde rice	535.5		-3.35	6.04
DIFPVP	11-S Seed Storage Protein. Plant	686.4	6	0.800	3.75
DIFPVPQ	11-S Seed Storage Protein. Plant	814.4	7	0.186	3.75
DIFPVPQF	11-S Seed Storage Protein. Plant	961.5	8	0.513	3.75
DLAIEK	Zinc finger. SWIM type. family	687.4	6	-0.133	4.18
DLGQEV	11-S Seed Storage Protein. Plant	659.3	6	-0.483	3.55
DLQIIRPPQ	11-S Seed Storage Protein. Plant	1078.6	9	-0.600	6.34
DLTGIPPAP	Heat Shock Protein Family 70	879.5	9	0.078	3.75

DLTGIPPAPR	Heat Shock Protein Family 70	1035.6	10	-0.380	6.34
DLTPEQ	Phythepsin	701.3	6	-1.500	3.55
DLYNPQ	11-S Seed Storage Protein. Plant	748.3	6	-1.600	3.75
DNVEEKKLVKYESDIDEP	Ribosomal protein Rsm22	2149.0	18	-1.483	3.96
DQEDEKPR	RmlC-like termite domain superfamily. rmlC-like jelly roll protein	1015.5	8	-3.438	4.11
DRGELRPN	11-S Seed Storage Protein. Plant	955.5	8	-2.213	6.50
DSFEGDE	11-S Seed Storage Protein. Plant	797.3	7	-1.771	3.29
DSTLIMQLLR	14-3-3 protein OS=Helianthus annuus	1188.7	10	0.480	6.34
DVAPPHGPL	Phythepsin	901.5	9	-0.233	5.29
DVNAAVATIK	Tubulin	1000.6	10	0.670	6.34
DWFNDGK	Transcription Factor bZIP Family	880.4	7	-1.843	4.11
DWILEEL	I-like periplasmic binding protein	916.5	7	0.100	3.42
DWLLEE	Transcription factor CG1-CAMTA family	803.4	6	-0.633	3.42
EALEPIE	11-S Seed Storage Protein. Plant	799.4	7	-0.286	3.47
EDLAALEK	Tubulin	887.5	8	-0.400	3.93
EFEGGSI	11-S Seed Storage Protein. Plant	737.3	7	-0.186	3.61
EFEGGSIE	11-S Seed Storage Protein. Plant	866.4	8	-0.600	3.47
EFEGGSIEH	11-S Seed Storage Protein. Plant	1003.4	9	-0.889	3.98
EFPPEE	RmlC-like termite domain superfamily. rmlC-like jelly roll protein	746.3	6	-1.817	3.47
EFPPEEE	RmlC-like termite domain superfamily. rmlC-like jelly roll protein	875.4	7	-2.057	3.37
EFVTPDEGQE	11-S Seed Storage Protein. Plant	1149.5	10	-1.320	3.33
EFVTPE	11-S Seed Storage Protein. Plant	720.3	6	-0.383	3.61
EFVTPEE	11-S Seed Storage Protein. Plant	849.4	7	-0.829	3.47
EFVTPEEE	11-S Seed Storage Protein. Plant	978.4	8	-1.163	3.37

EGQVVVIPQ	11-S Seed Storage Protein. Plant	967.5	9	0.511	3.85
EIIDLVDRL	PGG domain. superfamily of domains containing ankyrine repeats	1084.6	9	0.644	3.88
EKLPGGH	Dehydrin	736.4	7	-1.314	7.55
ELAPTHPI	14-3-3 protein	876.5	8	-0.063	5.36
EQQGEPW	11-S Seed Storage Protein. Plant	872.4	7	-2.414	3.61
ERAF	Raw black bean protein hydrolysates	521.6		-0.85	6.86
ERVIGDPE	Calcium-dependent channel. phosphate 7TM region	913.5	8	-1.038	3.93
ETEIQRP	2S Seed Storage Protein	871.4	7	-1.829	4.26
ETEIQRPV	2S Seed Storage Protein	970.5	8	-1.075	4.26
ETEIQRPVG	2S Seed Storage Protein	1027.5	9	-1.000	4.26
ETEIQRPVS	Bifunctional Inhibitor/Plant Lipid Transfer Protein/Helical Seed Storage	1057.5	9	-1.044	4.26
ETGRPGQSQRPS	11-S Seed Storage Protein. Plant	1298.6	12	-2.150	10.40
ETTL	Hazelnut protein hydrolysates	462.5		-0.275	0.92
EVDEQMINVQNK	Purine-nucleoside phosphorylase	1445.7	12	-1.133	3.93
FDDMIQE	11-S Seed Storage Protein. Plant	896.4	7	-0.686	3.38
FDDMIQEG	11-S Seed Storage Protein. Plant	953.4	8	-0.650	3.38
FDGAINVDITEFQTNLVPYPR	Tubulin	2408.2	21	-0.152	3.88
FDSGDQ	11-S Seed Storage Protein. Plant	667.2	6	-1.483	3.49
FEGGSI	11-S Seed Storage Protein. Plant	608.3	6	0.367	3.85
FEGGSIEH	11-S Seed Storage Protein. Plant	874.4	8	-0.563	4.25
FHVDQET	11-S Seed Storage Protein. Plant	874.4	7	-1.057	4.18
FKENIDNP	11-S Seed Storage Protein. Plant	975.5	8	-1.525	4.18
FKENIDNPS	11-S Seed Storage Protein. Plant	1062.5	9	-1.444	4.18
FLAGNPK	11-S Seed Storage Protein. Plant	745.4	7	-0.143	9.70
FNDEVQEQ	11-S Seed Storage Protein. Plant	1007.4	8	-1.750	3.42

FNGIEK	RmlC-like termite domain superfamily. rmlC-like jelly roll protein	706.4	6	-0.667	6.41
FNVDHE	11-S Seed Storage Protein. Plant	759.3	6	-1.117	4.18
FNVDHET	11-S Seed Storage Protein. Plant	860.4	7	-1.057	4.18
FPPEEDYG	RmlC-like termite domain superfamily. rmlC-like jelly roll protein	952.4	8	-1.575	3.42
FPPEEE	RmlC-like termite domain superfamily. rmlC-like jelly roll protein	746.3	6	-1.817	3.47
FPVPQ	11-S Seed Storage Protein. Plant	586.3	5	0.060	6.10
FREGDI	11-S Seed Storage Protein. Plant	735.4	6	-0.767	4.18
FREGDIIA	11-S Seed Storage Protein. Plant	919.5	8	0.213	4.18
FSVGGGDAE	RmlC-like cupin domain superfamily. rmlC-like jelly roll protein	837.4	9	-0.022	3.55
FVEQQMQQSPR	2S Seed Storage Protein	1376.7	11	-1.409	6.41
FVTPDEGQ	11-S Seed Storage Protein. Plant	891.4	8	-0.775	3.55
FVTPEEE	11-S Seed Storage Protein. Plant	849.4	7	-0.829	3.47
FVTPEEEQ	11-S Seed Storage Protein. Plant	977.4	8	-1.163	3.47
FVVPR	Uncharacterized protein	616.4	5	1.020	10.55
GDIFPV	11-S Seed Storage Protein. Plant	646.3	6	1.000	3.75
GDIFPVP	11-S Seed Storage Protein. Plant	743.4	7	0.629	3.75
GDIFPVPQ	11-S Seed Storage Protein. Plant	871.4	8	0.113	3.75
GDIFPVPQF	11-S Seed Storage Protein. Plant	1018.5	9	0.411	3.75
GDKLPIGPPS	Protein associated with the oily body	979.5	10	-0.550	6.34
GDRGDEDFDRDE	Uncharacterized protein	1424.5	12	-2.625	3.69
GELRPNA	11-S Seed Storage Protein. Plant	755.4	7	-1.129	6.41
GGSIEH	11-S Seed Storage Protein. Plant	598.3	6	-0.633	5.36
GGTGIGTGGHG	Dehydrin	869.4	11	-0.264	7.55
GHIVNVGQDL	11-S Seed Storage Protein. Plant	1050.5	10	0.220	5.29

GIDQEDEKPR	RmlC-like termite domain superfamily. rmlC-like jelly roll protein	1185.6	10	-2.340	4.11
GIPPAPR	Heat Shock Protein Family 70	706.4	7	-0.486	10.55
GIVSPH	11-S Seed Storage Protein. Plant	608.3	6	0.450	7.55
GQSQRPGWE	11-S Seed Storage Protein. Plant	1043.5	9	-2.122	6.41
GQSQRPGWETGRP	11-S Seed Storage Protein. Plant	1454.7	13	-2.023	10.40
GQSQRPGWETGRPEQ	11-S Seed Storage Protein. Plant	1711.8	15	-2.220	6.53
GYSFTTTAER	Actin family. ATPase. protein containing nucleotide binding domain	1131.5	10	-0.800	6.40
HGQSQRPGWET	11-S Seed Storage Protein. Plant	1281.6	11	-2.091	7.55
HGQSQRPGWETG	11-S Seed Storage Protein. Plant	1338.6	12	-1.950	7.55
IANLAG	11-S Seed Storage Protein. Plant	557.3	6	1.333	6.10
IANLN	11-S Seed Storage Protein. Plant OS	543.3	5	0.620	6.10
IDLAIE	Zinc finger. SWIM type. family FHY3/FAR1 OS	672.4	6	1.267	3.55
IDLAIEK	Zinc finger. SWIM type. family FHY3/FAR1 OS	800.5	7	0.529	4.18
IEALEPI	11-S Seed Storage Protein. Plant OS	783.4	7	0.857	3.61
IEALEPIE	11-S Seed Storage Protein. Plant OS	912.5	8	0.313	3.47
IFPVP	11-S Seed Storage Protein. Plant OS	571.3	5	1.660	6.10
IFPVPQ	11-S Seed Storage Protein. Plant OS	699.4	6	0.800	6.10
IGGIGTVPVGR	Elongation factor 1-alpha OS	1024.6	11	0.818	10.55
IINPR	11-S Seed Storage Protein. Plant OS	611.4	5	-0.120	10.55
IIRPPQ	11-S Seed Storage Protein. Plant OS	722.4	6	-0.367	10.55

IIVDDVNNPA	11-S Seed Storage Protein. Plant OS	672.4	10	0.360	3.59
IIVDVD	11-S Seed Storage Protein. Plant	1068.5	6	1.733	3.49
ILGDV	Phythepsin	515.3	5	1.720	3.75
IPTGTA	1-S Seed Storage Protein. Plant OS	558.3	6	0.483	6.10
IVDDVNNP	11-S Seed Storage Protein. Plant OS	884.4	8	-0.338	3.49
IVDDVNNPA	11-S Seed Storage Protein. Plant OS	955.5	9	-0.100	3.49
IVRPPQ	11S Seed Storage Protein Globulin G3 OS	708.4	6	-0.417	10.55
IVRPPQD	11S Seed Storage Protein Globulin G3 OS	823.5	7	-0.857	6.34
IWHHTFYNELR	Actin family. ATPase. protein containing nucleotide binding domain OS	1514.7	11	-0.882	7.71
KTVGGGDDAFNTFFSETGAGK	Tubulin	2105.0	21	-0.500	4.37
KVFPPEEQ	RmlC-like termite domain superfamily. rmlC-like jelly roll protein OS	972.5	8	-1.325	4.26
KVNIDNPSQAD	11S G3 Globulin Seed Storage Protein	1199.6	11	-1.209	4.11
LAGGI	11-S Vegetable Seed Storage Protein	429.3	5	1.860	6.10
LAGGIS	11-S Seed Storage Protein. Plant	516.3	6	1.417	6.10
LAVNLVPFPR	Purine-nucleoside phosphorylase	1124.7	10	0.940	10.55
LDLAGR	Actin family. ATPase. protein containing nucleotide-binding domain	643.4	6	0.167	6.34
LDVAPPHGPL	Phythepsin	1014.5	10	0.170	5.29
LEFEGGS	11-S Vegetable Seed Storage Protein	737.3	7	-0.286	3.61

LEFEGGSIEH	11-S Vegetable Seed Storage Protein	1116.5	10	-0.420	3.98
LGGTGTDHV	Dehydrin	855.4	9	-0.144	5.29
LIPEEQ	11-S Vegetable Seed Storage Protein	727.4	6	-0.633	3.61
LIVVPQ	11-S Vegetable Seed Storage Protein	667.4	6	1.933	6.10
LPLQDVYK	Elongation factor 1-alpha	974.5	8	-0.250	6.33
LQGIT	Glycoside hydrolase. family 28. pectin lyase/virulence factor	530.3	5	0.740	6.10
LQIIRPPQ	11-S Vegetable Seed Storage Protein	963.6	8	-0.238	10.55
LQSPDDT	11-S Vegetable Seed Storage Protein	774.3	7	-1.400	3.49
LVVVPQ	11-S Vegetable Seed Storage Protein	653.4	6	1.883	6.10
NALEPNE	11-S Vegetable Seed Storage Protein	785.4	7	-1.429	3.61
NDEVQEQ	11-S Vegetable Seed Storage Protein	860.4	7	-2.400	3.42
NDGQEEI	11-S Vegetable Seed Storage Protein	803.3	7	-1.914	3.42
NDGQEEIV	11-S Vegetable Seed Storage Protein	902.4	8	-1.150	3.42
NDLGQEV	11-S Vegetable Seed Storage Protein	773.4	7	-0.914	3.55
NDVSRP	11-S Vegetable Seed Storage Protein	686.3	6	-1.617	6.34
NDVSRPD	11-S Seed Storage Protein. Plant	801.4	7	-1.886	4.11
NGFTPE	11S Globulin G3 Seed Storage Protein	663.3	6	-1.150	3.85

NSDENEWFP	RmlC-like termite domain superfamily. rmlC-like jelly roll protein O	1136.4	9	-2.000	3.42
NSSYFVEWIPNNVK	Purine-nucleoside phosphorylase	1695.8	14	-0.543	6.40
PDEGQE	11-S Seed Storage Protein. Plant	673.3	6	-2.667	3.42
PFDED	11-S Seed Storage Protein. Plant	621.2	5	-1.860	3.38
PFGGQEE	11-S Seed Storage Protein. Plant O	762.3	7	-1.443	3.61
PGSGSISPI	11-S Seed Storage Protein. Plant	813.4	9	0.289	6.10
PGWETGRP	11-S Seed Storage Protein. Plant	898.4	8	-1.700	6.41
PGWETGRPE	11-S Seed Storage Protein. Plant	1027.5	9	-1.900	4.26
PILEHL	11-S Seed Storage Protein. Plant	720.4	6	0.633	5.36
PPAPR	Heat Shock Protein Family 70	536.3	5	-1.500	10.55
PPLTPTTRE	PGG domain-containing protein	1010.5	9	-1.233	6.41
PSFQPFP	11-S Seed Storage Protein. Plant	818.4	7	-0.500	6.10
PYYPNTPE	11-S Seed Storage Protein. Plant	979.4	8	-1.888	3.85
QFQPR	11-S Seed Storage Protein. Plant	674.4	5	-2.060	10.55
QIIRPP	11-S Seed Storage Protein. Plant	722.4	6	-0.367	10.55
QIIRPPQ	11-S Seed Storage Protein. Plant	850.5	7	-0.814	10.55
QIVRPPQ	11S Globulin G3 Seed Storage Protein	836.5	7	-0.857	10.55
QIVRPPQD	11S Globulin G3 Seed Storage Protein	951.5	8	-1.188	6.34
QLIVVPQ	11-S Seed Storage Protein. Plant	795.5	7	1.157	6.10
QLVVVPQ	11-S Seed Storage Protein. Plant	781.5	7	1.114	6.10
QSEIQRPV	Bifunctional Inhibitor/Plant Lipid Transfer Protein/Helical Seed Storage	955.5	8	-1.088	6.41
QSEIQRPVS	Bifunctional Inhibitor/Plant Lipid Transfer Protein/Helical Seed Storage	1042.5	9	-1.056	6.41
QSQRPGWET	11-S Seed Storage Protein. Plant	1087.5	9	-2.156	6.41
QVIQPPM	11-S Seed Storage Protein. Plant	811.4	7	0.057	6.10

QVVRPPI	11-S Seed Storage Protein. Plant	807.5	7	0.243	10.55
QVVVVPQ	11-S Seed Storage Protein. Plant	767.5	7	1.171	6.10
RGDIFPVPQ	11-S Seed Storage Protein. Plant	1027.5	9	-0.400	6.34
RSGQETPEEGS	11-S Seed Storage Protein. Plant	1175.5	11	-2.109	3.98
RSGQETPEEGSGN	11-S Seed Storage Protein. Plant	1346.6	13	-2.085	3.98
SDENEWFP	RmlC-like termite domain superfamily. rmlC-like jelly roll protein	1022.4	8	-1.813	3.42
SDTIQVS	Glycoside hydrolase. family 28. pectin lyase/virulence factor	748.4	7	-0.086	3.75
SEIQRPV	11-S Seed Storage Protein. Plant	827.5	7	-0.743	6.41
SEIQRPVS	Bifunctional Inhibitor/Plant Lipid Transfer Protein/Helical Seed Storage	914.5	8	-0.750	6.41
SETEIQRPV	Bifunctional Inhibitor/Plant Lipid Transfer Protein/Helical Seed Storage	1057.5	9	-1.044	4.26
SFEGDE	11-S Seed Storage Protein. Plant	682.2	6	-1.483	3.42
SGQETPEEGS	11-S Seed Storage Protein. Plant	1019.4	10	-1.870	3.47
SGQETPEEGSGN	11-S Seed Storage Protein. Plant	1190.5	12	-1.883	3.47
SGQTQRPSWE	11-S Seed Storage Protein. Plant	1174.5	10	-2.020	6.41
SGQTQRPSWETGRPGQSQRPS	11-S Seed Storage Protein. Plant	2326.1	21	-2.024	12.20
SPGSGSISPI	11-S Seed Storage Protein. Plant	900.5	10	0.180	6.10
SQRPGWE	11-S Seed Storage Protein. Plant	858.4	7	-2.171	6.41
SQRPGWETG	11-S Seed Storage Protein. Plant	1016.5	9	-1.811	6.41
SQRPGWETGRP	11-S Seed Storage Protein. Plant	1269.6	11	-2.036	10.40
SQRPGWETGRPE	11-S Seed Storage Protein. Plant	1398.7	12	-2.158	6.53
SQRPGWETGRPEQ	11-S Seed Storage Protein. Plant	1526.7	13	-2.262	6.53
SRGDIFPVPQ	11-S Seed Storage Protein. Plant	1114.6	10	-0.440	6.34
SVSVPGDL	RmlC-like termite domain superfamily. rmlC-like jelly roll protein	772.4	8	0.638	3.75

TEIQRPVS	Bifunctional Inhibitor/Plant Lipid Transfer Protein/Helical Seed Storage	928.5	8	-0.738	6.41
TEPRLQ	Calcium-dependent channel. phosphate 7TM region	742.4	6	-1.667	6.41
TGQSQRPGWE	Calcium-dependent channel. phosphate 7TM region	1144.5	10	-1.980	6.41
TGQSQRPGWET	11-S Seed Storage Protein. Plant	1245.6	11	-1.864	6.41
TGQSQRPGWETGRP	11-S Seed Storage Protein. Plant	1555.7	14	-1.929	10.40
TGQSQRPGWETGRPE	11-S Seed Storage Protein. Plant	1684.8	15	-2.033	6.53
TGQSQRPGWETGRPEQ	11-S Seed Storage Protein. Plant	1812.9	16	-2.125	6.53
TLGIET	Heat Shock Protein Family 70	632.3	6	0.500	3.85
TTPSYVAFTDTER	Heat Shock Protein Family 70	1486.7	13	-0.708	4.18
VAPEEHPVLLTEAPLNPK	Actin family. ATPase. protein containing nucleotide binding domain	1953.1	18	-0.267	4.47
VEQQIQSSRP	Bifunctional Inhibitor/Plant Lipid Transfer Protein/Helical Seed Storage	1170.6	10	-1.300	6.41
VETGVIKPG	Elongation factor 1-alpha	898.5	9	0.267	6.41
VFPPEEDYG	Elongation factor 1-alpha	1051.4	9	-0.933	3.42
VFPPEEEDE	RmlC-like termite domain superfamily. rmlC-like jelly roll protein	1089.5	9	-1.522	3.26
VFPPEEEEDEYG	RmlC-like termite domain superfamily. rmlC-like jelly roll protein	1309.5	12	-1.575	3.19
VFPPEEEQ	RmlC-like termite domain superfamily. rmlC-like jelly roll protein	844.4	7	-0.957	3.61
VIQNLPNQ	2S Seed Storage Protein	924.5	8	-0.388	6.10
VIVDDV	11-S Seed Storage Protein. Plant	658.4	6	1.683	3.49
VLIPEEQ	11-S Seed Storage Protein. Plant	826.4	7	0.057	3.61

VNEGDV	11-S Seed Storage Protein. Plant	631.3	6	-0.417	3.55
VTPEEE	11-S Seed Storage Protein. Plant	702.3	6	-1.433	3.47
VVIPQ	11-S Seed Storage Protein. Plant	653.4	5	1.560	6.10
VVRPPI	11-S Seed Storage Protein. Plant	679.4	6	0.867	10.55
VVVIPQ	11-S Seed Storage Protein. Plant	554.3	6	2.000	6.10
VVVPQ	11-S Seed Storage Protein. Plant	540.3	5	1.500	6.10
VVVVPQ	11-S Seed Storage Protein. Plant	639.4	6	1.950	6.10
WDSFEGDE	11-S Seed Storage Protein. Plant	983.4	8	-1.663	3.29
WETGRPEQ	11-S Seed Storage Protein. Plant	1001.5	8	-2.325	4.26
WETGRPGQ	11-S Seed Storage Protein. Plant	929.4	8	-1.938	6.41
WETGRPGQSQ	11-S Seed Storage Protein. Plant	1144.5	10	-1.980	6.41
WHQHEQQPR	11-S Seed Storage Protein. Plant	1244.6	9	-3.044	7.71
YAWVLDK	Elongation factor 1-alpha	893.5	7	0.029	6.33
YGIVSP	11-S Seed Storage Protein. Plant	634.3	6	0.767	6.09
YGIVSPH	11-S Seed Storage Protein. Plant	771.4	7	0.200	7.54
YNDGQEE	11-S Seed Storage Protein. Plant	853.3	7	-2.743	3.42
YQMSPEQ	11-S Seed Storage Protein. Plant	881.4	7	-1.757	3.85
YRPGTVALR	Hap3/NF-YB family transcription factor	1031.6	9	-0.356	11.15
YSDQQQQRP	Transcription factor ssDNA-binding-TF family	1148.5	9	-2.856	6.33
YVEQQIQSSRP	Bifunctional Inhibitor/Plant Lipid Transfer Protein/Helical Seed Storage	1333.7	11	-1.300	6.40
YVTEGA	11-S Seed Storage Protein. Plant	638.3	6	0.017	3.85

Table S4. SPD 1.0 mg/mL basolateral compartment.

Sequence	Origen	Molecular Weight	N° of AA	Hydrophobicity	Isoelectric Point
AFVHWYVGEGMEEGEFSEAR	Tubulin	2329.0	20	-0.495	4.02
AGPYGQIFRPDNFVFGQSGAGNNWAK	Purine-nucleoside phosphorylase	2797.3	26	-0.581	9.30
AIFVDLEPTVIDEVR	Tubulin	1714.9	15	0.613	3.74
AVFVDLEPTVIDEVR	Tubulin	1700.9	15	0.593	3.74
DAHK	Albuminde rice	469.5	4	-2.2	7.83
DVNAAVATIK	Tubulin	1000.6	10	0.670	6.34
EIQTAVR	Hap3/NF-YB family transcription factor	815.5	7	-0.243	6.41
EIVDLCLDR	Tubulin	1131.6	9	0.422	3.88
EVDEQMLNVQNK	Tubulin's beta-2 chain	1445.7	12	-1.192	3.93
FDLMYAK	Tubulin	886.4	7	0.229	6.33
FPGQLNSDLR	Purine-nucleoside phosphorylase	1145.6	10	-0.740	6.34
GHYTIGK	Tubulin	774.4	7	-0.771	9.30
GILTLK	Actin family. ATPase. protein containing nucleotide binding domain	643.4	6	1.183	9.70
GYSFTTTAER	Actin family. ATPase. protein containing nucleotide binding domain	1131.5	10	-0.800	6.40
IINEPTAAAIAYGLDK	Heat Shock Protein Family 70	1658.9	16	0.381	4.18
IWHHTFYNELR	Actin family. ATPase. protein containing nucleotide binding domain	1514.7	11	-0.882	7.71

LDLQR	Defatted wheat germ powder	643.7	5	-0.78	6.64
LIGDAAK	Heat Shock Protein Family 70	686.4	7	0.586	6.34
LSTIR	Tubulin	588.4	5	0.460	10.55
LSVDYGK	Tubulin	780.4	7	-0.271	6.33
MEMK	Hydrolysates of marine bivalves	537.7	4	-0.9	6.61
NMMCAADPR	Purine-nucleoside phosphorylase	1064.4	9	-0.356	6.16
NNIANDPGCEVILSNHIKEALER	Germacrena D sintase	2548.3	23	-0.478	4.58
NSSYFVEWIPNNVK	Purine-nucleoside phosphorylase	1695.8	14	-0.543	6.40
PLPR	Albuminde rice	481.6	4	-0.975	11.29
QLFHPEQLISGK	Tubulin	1395.8	12	-0.458	7.55
SIEIQAPTGEIIK	Family of CCHC(Zn) transcription factor interactors and regulators	1397.8	13	0.146	4.26
STAGDTHLGGEDFDNR	Heat Shock Protein Family 70	1690.7	16	-1.263	4.06
TIQFVDWCPTGFK	Tubulin	1597.8	13	0.123	6.16
TTPSYVAFTDTER	Heat Shock Protein Family 70	1486.7	13	-0.708	4.18
TVQFVDWCPTGFK	Tubulin	1583.7	13	0.100	6.16
VAPEEHPVLLTEAPLNPK	Actin family. ATPase. protein containing nucleotide binding domain	1953.1	18	-0.267	4.47
VEIIANDQGNR	Heat Shock Protein Family 70	1227.6	11	-0.673	4.18
YFPTQALNFAFK	Mitochondrial transporter protein	1445.7	12	0.108	9.30

YLLLK	Palm kernel cake hydrolysates	648.8	5	1.24	9.49
YLVR	Hazelnut protein	549.7	4	0.55	9.57