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Fast Skeletal Muscle Troponin and Tropomyosin as a Dietary Source of Antidiabetic and Antihypertensive Bioactive Peptides: An In Silico Study

Troponina y Tropomiosina de Músculo Esquelético como Fuente Alimentaria de Péptidos Bioactivos Antidiabéticos y Antihipertensivos: Estudio In Silico

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ABSTRACT

Background: The nutraceutical properties of food hydrolysates rely on multiple biochemical interactions involving the modulation of enzymes and cellular receptors. Numerous bioactive peptides released from troponin and tropomyosin digestion have been identified. Their characterization has mostly been performed by hydrolysis catalyzed by proteases unrelated to the human digestive system. **Objective:** This study aimed to determine the bioactive profile of beef, pork, and chicken meat by analyzing the frequency and pharmacokinetics of biopeptides released from troponin and tropomyosin. **Methods:** *In silico* digestion and biopeptide release frequency were studied by three parameters; bioactive fragments release frequency (A_E), frequency percentage (W), and mean occurrence (A_S), all stated on the BIOPEP-UWM platform. Further on, hydrolysis end-products were screened based on gastrointestinal-absorption probability and pharmacokinetic profiling performed on SwissADME, SwissTargetPrediction, and ADME/Tlab bioinformatics web tools. Statistical analyses were performed using a one-way ANOVA test. **Results:** Dipeptidyl peptidase-IV (DPP-IV) and angiotensin-converting enzyme (ACE) inhibiting biopeptides exhibited the highest release frequency. Moreover, W and A_S parameters showed no significant difference ($p>0.05$) between the myofibrillar isoforms assessed. Seven biopeptides were classified as highly absorbable and reported optimal drug-likeness compliance. Although biopeptides hold good pharmacokinetic properties, the therapeutic potency of biopeptides showed to be lower than those of DPP-IV and ACE-inhibiting drugs. **Conclusions:** Troponin and tropomyosin are rich dietary sources of bioactive peptides, mainly DPP-IV and ACE inhibitors. Digestion end-products are mainly dipeptides with optimal pharmacokinetic and drug-like properties, suggesting a potential therapeutic application in hypertensive and hyperglycemic disorders.

Keywords: Bioactive peptides; angiotensin-converting enzyme inhibitors; dipeptidyl-peptidase IV inhibitors; Tropomyosin; Troponin.

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RESUMEN

Antecedentes: Las propiedades nutracéuticas de los hidrolizados de alimentos dependen de múltiples interacciones bioquímicos que involucran la modulación de enzimas y receptores celulares. Se han identificado numerosos péptidos bioactivos liberados de la digestión de troponina y tropomiosina, pero su caracterización se ha llevado a cabo principalmente por hidrólisis catalizada por proteasas ajenas al sistema digestivo humano. **Objetivo:** Este estudio tuvo como objetivo determinar el perfil bioactivo de la carne de res, cerdo y pollo mediante el análisis de la frecuencia y farmacocinética de los biopéptidos liberados de la troponina y la tropomiosina. **Métodos:** Se estudió la digestión *in silico* y la frecuencia de liberación de biopéptidos mediante dos parámetros; frecuencia de liberación de fragmentos bioactivos (A_E), frecuencia porcentual (W) y ocurrencia media (A_S), ambos indicados en la plataforma BIOPEP-UWM. Más adelante, los productos finales de la hidrólisis se examinaron en función de la probabilidad de absorción gastrointestinal y el perfil farmacocinético realizado en las herramientas bioinformáticas SwissADME, SwissTargetPrediction y ADME/Tlab. El análisis estadístico se llevó a cabo mediante una prueba ANOVA de una vía. **Resultados:** Los biopéptidos inhibidores de la dipeptidil peptidasa IV (DPP-IV) y la enzima convertidora de angiotensina (ECA) exhibieron la mayor frecuencia de liberación. Además, los parámetros W y A_S no mostraron diferencias significativas ($p > 0.05$) entre las isoformas miofibrilares evaluadas. Siete biopéptidos se clasificaron como altamente absorbibles e informaron un cumplimiento óptimo de similitud con el fármaco. Aunque los biopéptidos tienen propiedades farmacocinéticas adecuadas, su potencia terapéutica demostró ser menor que la de los fármacos inhibidores de la DPP-IV y la ACE. **Conclusiones:** La troponina y la tropomiosina son una fuente dietética rica en péptidos bioactivos, principalmente DPP-IV e inhibidores de la ACE. Los productos finales de la digestión son principalmente dipéptidos con propiedades farmacocinéticas óptimas y similares a la de los fármacos, lo que sugiere una aplicación terapéutica factible en trastornos hipertensivos e hiperglicémicos.

Palabras clave: Péptidos bioactivos; Inhibidores de la enzima convertidora de angiotensina; inhibidores de la dipeptidil-peptidasa IV; Tropomiosina; Troponina.

INTRODUCTION

Meat is ranked as one of the most important foodstuffs on a daily-basis diet, being a rich source of minerals, fatty acids, and both essential and non-essential amino acids (1). Among the wide range of polypeptides present within the muscle fibers, myofibrillar proteins account for more than 50% of the protein content in meat (2). Two of these contractile proteins hold significant physiological functions; troponin, a globular protein with three subunits (T, I, and C), and tropomyosin, a fibrillar polypeptide made up of α and β chains. These myofibrillar proteins are scattered across sarcomeres along the muscle fibers; thus, besides their well-established role in muscle contraction, they represent a major source of dietary amino acids when digested in the gastrointestinal tract.

Growing research in food chemistry has revealed the therapeutic effects of nutrients, currently known as nutraceutical properties (3). Nutraceuticals exhibit a broad spectrum of health-promoting properties, including vascular resistance reduction, glycemic regulation, and free-radicals scavenging, and have also shown to be beneficial in numerous metabolic disorders (3). Dietary protein intake, and myofibrillar proteins, particularly, are a rich source of biologically active molecules known as bioactive peptides (4). The biological properties of these oligopeptides rely on several mechanisms involving the modulation of enzymes and cellular receptors. Recent trends in biopeptides research have led to the development of in silico tools that enable a simulated/computerized enzymatic hydrolysis of polypeptides helping to characterize the bioactive products from protein digestion (5) relative frequency of release of fragments with a given activity by selected enzyme(s. As a result, this bioinformatic approach has allowed researchers to determine the profile of bioactive peptides released from dietary protein when hydrolyzed by specific subsets of enzymes.

As stated by Wang *et al*. (6), *in silico* experimentation has proved to be a useful approach to predict the bioactive profile, particularly dipeptidyl-peptidase IV (DPP-IV) inhibitory properties, of protein hydrolysates. Although *in vivo* studies are limited, they provide valuable evidence that DPP-IV inhibiting biopeptides from dietary protein intake improve glycemic regulation and enhance insulin sensitivity in diabetic animal models (7). Consequently, the bioactive properties of food-hydrolysates have been thoroughly investigated, regarding the pharmacokinetics and drug-likeness of these nutraceuticals are still scarce (8). Pharmacokinetic traits must be revealed to elucidate the therapeutic potential of biopeptides; however, studying the pharmacological parameters of bioactive peptides

would require extensive laboratory research and clinical trials. Fortunately, bioinformatic-assisted experimental designs allow the evaluation of compounds based on molecular and topological descriptors (9); for instance, absorption, distribution, excretion, metabolism, and toxicity (ADEM/T) (10) and target prediction analysis (11) enable to develop of a pharmacokinetic characterization from a chemoinformatic theoretical approach which help to elucidate biopeptides pharmacological traits.

Many bioactive peptides have been identified, and their isolation has been performed by enzymatic proteolysis catalyzed by a wide range of proteases unrelated to the human digestive system. This study aimed to characterize the bioactive peptides obtained from beef, pork, and chicken meat when subjected to an *in silico* digestion by pepsin (gastric digestion), trypsin, and chymotrypsin (pancreatic digestion). We expected to identify the difference in biopeptide-release frequency between the isoforms of these species and filter the obtained bioactive peptides based on their drug-likeness compliance and pharmacokinetic profile. Screening methods carried out through an *in silico* approach serve to identify suitable meat-hydrolysates with a therapeutic potential before *in vitro* and *in vivo* experimentation.

MATERIALS AND METHODS

Sequence retrieval

Aiming to analyze the bioactive peptides released from beef (cow - *Bos taurus*), chicken meat (*Gallus gallus*), and pork (pig - *Sus scrofa*) meat digestion, the sequences from fast skeletal muscle troponin subunits (troponin C, troponin T, and troponin I) and tropomyosin subunits (tropomyosin-α chain and tropomyosin-β chain) were retrieved from the UniProt database (https://www.uniprot.org) (12). For each species, five peptidic sequences were obtained (Table 1).

Table 1. Data from the proteins retrieved from the UniProt database.

Organism	Protein	Amino acids length	Entry code (ID)
Bos taurus	Troponin C	161	Q148C2|Q148C2_BOVIN
	Troponin T	270	Q8MKI3|TNNT3_BOVIN
	Troponin I	178	F6QIC1|F6QIC1_BOVIN
	Tropomyosin-α	284	Q5KR49|TPM1_BOVIN
	Tropomyosin-β	284	Q5KR48|TPM2_BOVIN
Gallus gallus	Troponin C	163	P02588|TNNC2_CHICK
	Troponin T	263	P12620|TNNT3_CHICK
	Troponin I	183	P68246|TNNI2_CHICK
	Tropomyosin-α	284	P04268|TPM1_CHICK
	Tropomyosin-β	284	P19352|TPM2_CHICK
Sus scrofa	Troponin C	159	P02587|TNNC2_PIG
	Troponin T	271	Q75NG9|TNNT3_PIG
	Troponin I	182	Q4JH15|Q4JH15_PIG
	Tropomyosin-α	248	A0A4X1TTA0|A0A4X1TTA0_PIG
	Tropomyosin-β	287	A1X899|A1X899_PIG

Bioactivity profiling

In silico enzymatic digestion and bioactivity profiling were analyzed using the BIOPEP-UWM database (13) especially on these derived from foods and being constituents of diets that prevent development of chronic diseases. The database is continuously updated and modified. The addition of new peptides and the introduction of new information about the existing ones (e.g., chemical codes and references to other databases) and served to evaluate troponin and tropomyosin as a source of

bioactive peptides. Gastrointestinal digestion was simulated through *in silico* proteolysis catalyzed by enzymes involved in gastric and pancreatic digestion in the human digestive system; pepsin (E.C 3.4.23.1) [pH<2], trypsin (E.C 3.4.21.4), and chymotrypsin (E.C 3.4.21.1). The bioactive profile was assessed for each polypeptide and later on compared between species according to the parameters stated by Minkiewicz *et al.* (13). The frequency of release of bioactive fragments with given activity in a protein sequence (A_E) was determined using the equation 1:

$$A_E = d/N \quad [1]$$

where "d" is the number of peptides with specific activity (e.g., DPP-IV inhibitors) and "N" is the number of residues in the sequence.

Further on, the relative frequency percentage of fragments release (W) was calculated as stated in equation 2 based on a (total number of released biopeptides with given activity) and the results obtained from equation 1.

$$A_S = a_t/N_t \quad [2]$$

At last, the bioactive profile of the whole set of proteins derived from a single species was compared. The mean occurrence of bioactive fragments in a set of proteins (A_S) was calculated using equation 3:

$$A_S = a_t/N_t \quad [3]$$

where "a_t" represents the total number of bioactive peptides with a given activity, and "N_t" is the number of amino acids in the whole set of proteins.

The parameter A_S enabled a comparative analysis of the bioactive potential of the proteins between the species under study. Statistical analysis was conducted to determine a significant difference in the bioactive profile of troponin and tropomyosin derived from each of the three species. A one-way ANOVA test was applied with a significance level of 0.05.

Therapeutic potential assessment

Biopeptides obtained from meat digestion were screened to evaluate the therapeutic properties based on gastrointestinal absorption probability (GI-absorption) determined through the SwissADME web tool (14) and ADME/T lab platform (10). Those peptides classified as GI-absorbable in both web tools were subjected to pharmacokinetic profiling, drug-likeness analysis, and target prediction. The compliance with Lipinski's rule of 5, Ghose, Veber, Egan, and Muegge drug-likeness rules were assessed.

Absorption, distribution, metabolism, excretion, and toxicity (ADME/T) parameters were predicted for the GI-absorbable biopeptides intending to develop a pharmacokinetic profile for each molecule. The target proteins for these peptides were determined on the SwissTarget-Prediction platform by searching for the binding affinity for specific receptors/enzymes (11). Statistical analyses were performed using a one-way ANOVA test with a significance level of 0.05.

RESULTS AND DISCUSSION

Troponin and Tropomyosin bioactive profile

A simulated gastric and pancreatic digestion was performed *in silico*, resulting in 40 bioactive peptides released from all the myofibrillar proteins isoforms analyzed. The bioactive profile of troponin and tropomyosin subunits from each species was assessed based on the biopeptide release frequency. The relative frequency percentage of released fragments (W) was determined (Figures 1 and 2). The bioactive profile of the set of proteins from a single species was analyzed based on the A_S parameter (Figure 3).

The bioactive profile of troponin subunits, assessed by the W parameters, revealed no significant difference ($p>0.05$) between the isoforms of the species under study (Figure 1). Seven classes of bioactive peptides were reported: angiotensin-converting enzyme (ACE) inhibitors, antioxidants, stimulating, dipeptidyl-peptidase IV (DPP-IV) inhibitors, calmodulin-dependent phosphodiesterase (CaMPDE) inhibitors, renin inhibitors, and dipeptidyl-peptidase III (DPP-III) inhibitors.

Two types of bioactive peptides were the most frequently released upon enzymatic digestion: DPP-IV inhibitors (DPP-IVi) and ACE inhibitors (ACEi). As presented in Figure 1, troponin T isoforms showed a release frequency of DPP-IVi >34.0%, with the highest value attributed to pork (pig) (40.2%). The release frequency of DPP-IVi for troponin I was greater than 40.0%, and chicken reported the highest frequency among all three species (63.2%). At last, the troponin C release frequency of DPP-IVi was higher than 24.0% for all three species, and beef (cow) exhibited the highest score (36.8%).

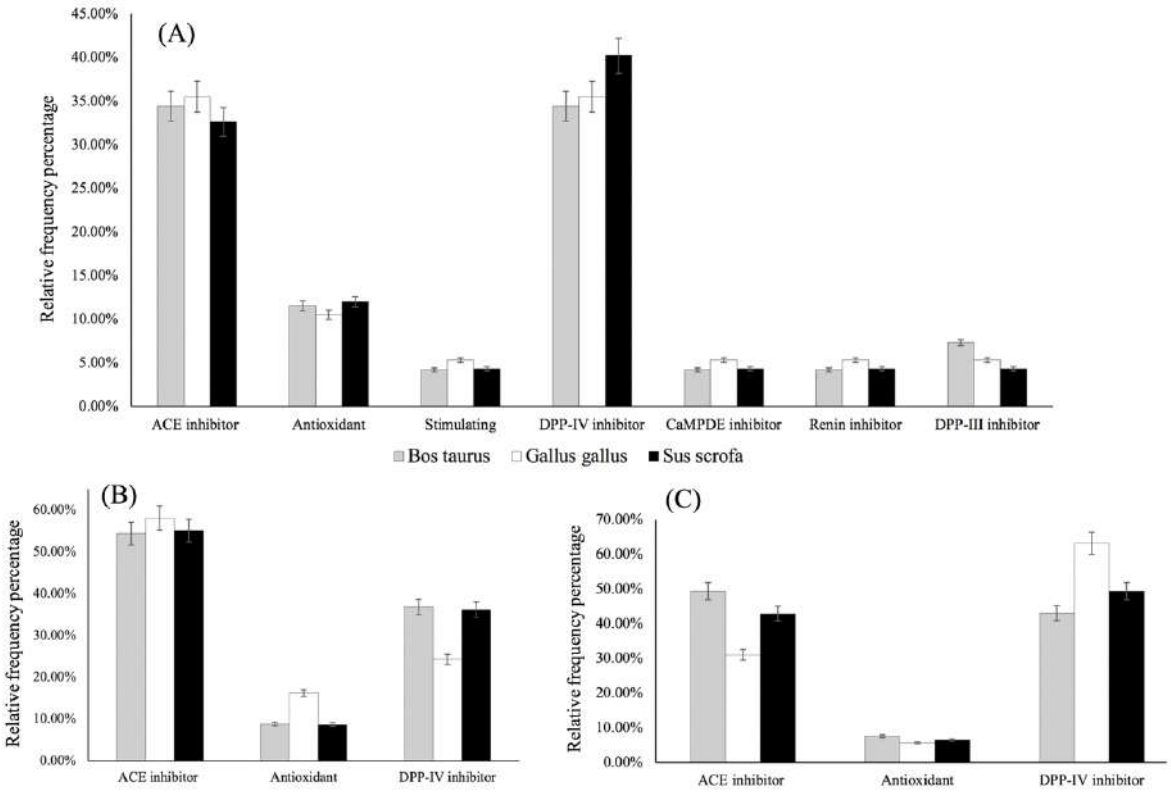

Figure 1. Relative frequency percentage of bioactive peptides released from troponin. (A) Bioactive profile of troponin T. (B) Bioactive profile of troponin C. (C) Bioactive profile of troponin I.

DPP-IV is a serine protease involved in the degradation of incretin hormones, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) (15). These hormones exert diverse physiological functions, including insulin secretion potentiation following food intake, slowing gastric emptying, and satiety stimulation (15). Thus, DPP-IV inhibition is a suitable therapeutic approach for glycemic regulation in hyperglycemic disorders. The high release frequency values of DPP-IVi bioactive peptides suggest that troponin and tropomyosin dietary intake hold therapeutic properties for glycemia reduction. Although these outcomes are mainly theoretical, *in vivo* experimentation supports the results of this study, as stated by Casanova-Martí *et al.* (16), who found that biopeptides derived from chicken had a high DPP-IV inhibitory capacity and improved GLP-1 secretion and glucose tolerance in rats.

In addition to DPP-IVi, ACE inhibitors were the second most frequently obtained bioactive peptides (Figure 1). Among the products of troponin C digestion, ACEi was the most commonly released (>54.0%); and chicken isoforms showed the highest W value from all three species (58.1%). The frequency of ACEi biopeptides release from troponin T was above 32.0%, and similar to troponin C, the chicken isoform scored the highest (35.5%). On the contrary, W parameter analysis of troponin I hydrolysis showed that the chicken isoform had the lowest ACEi release frequency (31.0%), while the other two were above 42.0%.

ACE is a carboxypeptidase that catalyzes the activation of angiotensin II. It is one of the main hormones of the renin-angiotensin-aldosterone system involved in fluid retention by aldosterone secretion stimulation and blood pressure increase by induced vascular spasm (17). ACE inhibition has long been known for its favorable outcomes in hypertension therapy. Bioactive peptides such as Val-Tyr and Ile-Trp have shown vascular resistance modulation (18). However, several biopeptides lack *in vitro* evidence of ACE inhibition and blood pressure reduction. Thus, *in vivo* screening studies are still required to determine suitable anti-hypertensive properties when orally delivered.

Antioxidant biopeptides were obtained from the troponin isoforms of all three species (Figure 1). Results varied irregularly. Troponin T isoforms showed a release frequency (W) greater than 10.0% and pork (pig) exhibited the highest value (12.00%). Troponin C revealed values greater than 8.0%, with the chicken isoform exhibiting more than twice the

value of the two other isoforms (16.2%). Finally, troponin I presented a release frequency greater than 5.0%, with beef (cow) scoring the highest value (7.6%). While antioxidant bioactive peptides released from meat digestion hold diverse functions, the peptides obtained act mainly by free radicals scavenging and oxidative stress reduction (19, 20). Moreover, DPP-III inhibitors (DPP-IIIi) were reported as hydrolysis products of troponin T exclusively. The release frequency (W) of this biopeptide was above 4.0% for all three species, and beef (cow) showed the highest score (7.3%). Results suggest a dietary intake of DPP-IIIi biopeptides. However, while DPP-III inhibition has shown improved hemodynamics in rodents (21), the role of DPP-III bioactive peptides as hemodynamic enhancers awaits to be unrevealed.

Troponin T was the only source of stimulating biopeptides, calmodulin-dependent phosphodiesterase (CaMPDE) inhibitors, and renin blockers. For these three classes of biopeptides, the release frequency (W) was higher than 4.0%, with the chicken isoform slightly above the other two (5.3%) (Figure 1). Stimulating peptides enhance glucose uptake (22); however, its mechanism is not entirely understood. CaMPDE is a phosphodiesterase involved in the cross-talk between cAMP and Ca^{2+} signaling in a wide range of cell populations (23). CaMPDE inhibitors have been shown to exert anti-inflammatory effects on glial cells and could be therapeutic in neurodegenerative disorders (24). Renin-inhibitors appear to block renin's cleavage from angiotensinogen to angiotensin I. Despite the poor outcomes of renin-inhibiting drugs for hypertension therapy (25), bioactive peptides derived from meat digestion could interact synergically with commonly administered drugs for hypertension therapy.

Similar to the bioactive profile of troponin subunits, no significant difference was found in biopeptides release frequency ($p>0.05$) between the tropomyosin isoforms (Figure 2). Tropomyosin-α exhibits the highest number of bioactive peptides released upon gastric and pancreatic digestion, and except for pig's isoform, the most frequently released biopeptides were DPP-IV inhibitors.

Tropomyosin-β showed a high bioactive profile for DPP-IVi biopeptides release; however, only three classes of bioactive peptides were obtained from its digestion (Figure 2). For DPP-IVi, a release frequency >50.0% was reported, and beef (cow) seems to be the isoform that is a better precursor of these peptides. ACEi release frequency was highest for the chicken isoform (36.7%), and antioxidant biopeptides were more frequently released from the pig's isoform (17.9%).

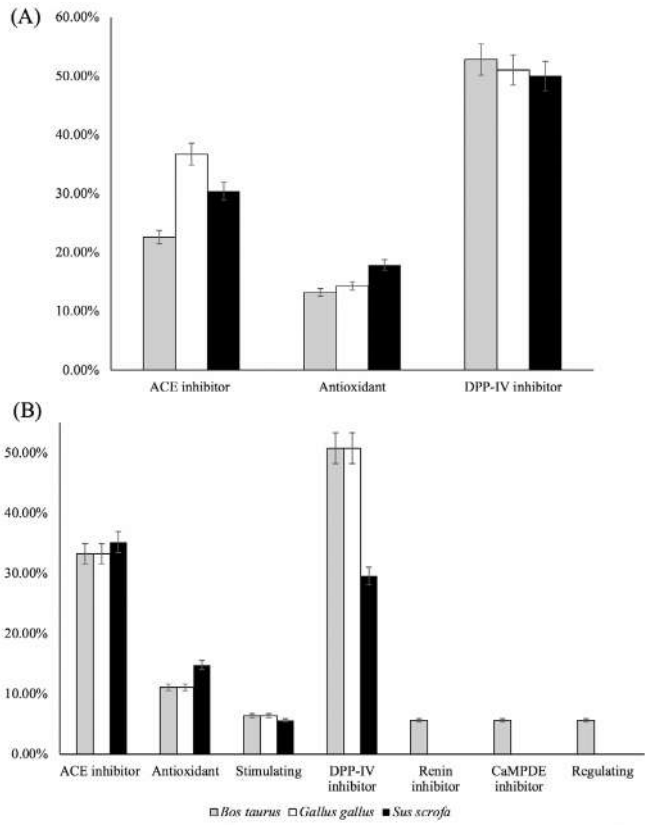

Figure 2. Relative frequency percentage of bioactive peptides release from tropomyosin. (A) Bioactive profile of tropomyosin-β. (B) Bioactive profile of tropomyosin-α.

On the other hand, tropomyosin-α reported being a rich source of ACEi biopeptides with a release frequency greater than 33.0% and with pig's isoform scoring the highest value (35.2%), The release frequency of DPP-IVi biopeptides was >50.0% for beef (cow) and chicken, and <30.0% for pork (pig). On the other hand, antioxidant bioactive peptides reported a release frequency of over 10.0%, and pig's isoform exhibited the highest value (14.8%). Stimulating peptides, renin inhibitors, CaMPDE inhibitors, and regulating biopeptides all showed a release frequency lower than 7.0%.

Most of the biological activities reported in tropomyosin digestion end-products were also identified in dipeptides released from troponin digestion, except for the regulating biopeptides. Dipeptide with sequence Asp-Tyr was the only regulating biopeptide obtained purely from beef (cow) tropomyosin-α. This bioactive peptide exhibits a regulation of ionic flux across the cell membrane, showing electrophysiological modulation on neurons in rodents (26).

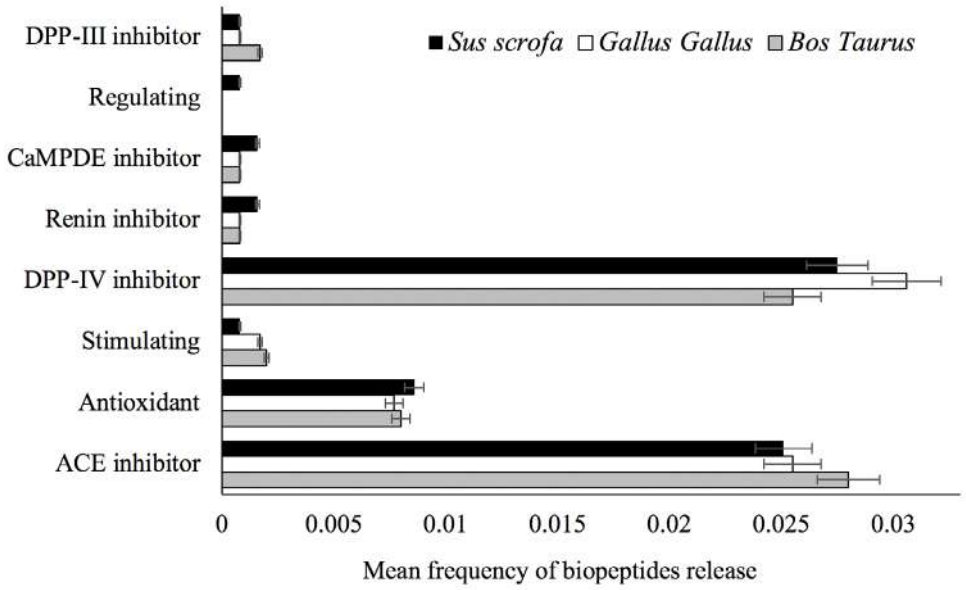

Figure 3. Mean frequency of occurrence of bioactive peptides in troponin and tropomyosin from beef (*B. taurus*), chicken (*G. gallus*) and pork (*S. scrofa*) fast skeletal muscle.

The bioactive profile of the myofibrillar proteins from the three species revealed no significant difference ($p>0.05$) in terms of the mean frequency (AS) of bioactive peptides occurrence (Figure 3). Nonetheless, variations were observed regarding the biological activity of the biopeptides obtained. DPP-IVi peptides were most frequently released from chicken (*G. gallus*) and pig (*S. scrofa*) troponin and tropomyosin. The findings of Martini *et al.* (27) support the results of this study, as they found out by in vitro experimentation that pork had a higher DPP-IV inhibiting activity than chicken meat, yet all the types of meat analyzed (pork, beef, chicken, and turkey) were determined to be good sources of DPP-IVi bioactive peptides. On the other hand, troponin and tropomyosin isoforms from the cow exhibited the highest release frequency of ACEi biopeptides from all three species. Similarly, Mora *et al.* (28) analyzed meat proteins and concluded that meat is a rich source of peptides with ACE inhibitory properties.

Antioxidant, stimulating, renin-inhibiting, CaMPDE-inhibiting, and DPP-III-inhibiting bioactive peptides reported a low mean frequency (A_S) of bioactive peptides occurrence when compared to DPP-IVi and ACEi peptides. Regulating peptides, on the other hand, were only obtained from beef. Compared to DPP-IV and ACE inhibition, these functions have been less studied and still need to be revealed.

Bioactivity and drug-like properties of GI-absorbable biopeptides

The biopeptides obtained from gastric and pancreatic simulated digestion were screened based on the classification as gastrointestinal-absorbable (GI-absorbable) biopeptides in SwissADME and ADME/Tlab platforms. The bioactivity and half-maximal effective concentration for DPP-IV inhibition (EC50) of GI-absorbable peptides are shown in Table 2.

Out of 40 bioactive peptides released from enzymatic proteolysis of troponin and tropomyosin isoforms, 7 were predicted to be easily absorbed by enterocytes. Likely absorbable dipeptides were obtained from all three species' isoforms except for three peptides. Ale-Phe and Cys-Phe were obtained only from *G. gallus* troponin C, and Gly-Leu was exclusively released from *S. scrofa* tropomyosin-β digestion. Hence, among the species under study, *G. gallus* proteins appear to be a better source of absorbable bioactive peptides. Ryan et al. (34) showed that chicken breast muscle is a rich source of several bioactive peptides; however, the evaluation of bioactive fragments release was performed with enzymes foreign to the human digestive system.

Table 2. Bioactivity of meat-derived GI-absorbable bioactive peptides.

Biopeptide Sequence	Action(s)	Source	EC_{50} (μM)	Reference
Val-Leu	(1) Glucose uptake stimulating (2) DPP-IV inhibitor	*B. taurus*: Tm-α, TnT *G. gallus*: Tm-α, TnT *S. scrofa*: Tm-α, TnT	(1): - (2): 74.00	(22, 29)
Ala-Leu	DPP-IV inhibitor	*B. taurus*: Tm-α, Tm-β, TnT *G. gallus*: Tm-α, Tm-β, TnT, TnI *S. scrofa*: TnT, TnI	882.1	(30)
Pro-Leu	ACE inhibitor	*B. taurus*: TnT *G. gallus*: TnT *S. scrofa*: TnT	337.3	(31)
Ile-Phe	ACE inhibitor	*B. taurus*: TnC *G. gallus*: TnC *S. scrofa*: TnC	930.0	(32)
Ala-Phe	ACE inhibitor	*G. gallus*: TnC	190.0	(32)
Cys-Phe	ACE inhibitor	*G. gallus*: TnC	2.0	(33)
Gly-Leu	(1) ACE inhibitor (2) DPP-IV inhibitor	*S. scrofa*: Tm-β	(1): 2500.0 (2): 2615.0	(30, 32)

DPP-IV, Dipeptidyl-Peptidase IV; ACE, Angiotensin Converting Enzyme; Tm-α, Tropomyosin-α; Tm-β, Tropomyosin-β; TnT, Troponin T; TnC, Troponin C; TnI, Troponin I; EC50, Half maximal effective concentration.

Two main properties were identified from the bioactive profile of the GI-absorbable biopeptides released: anti-diabetic and anti-hypertensive. DPP-IV inhibitors and glucose uptake stimulating biopeptides are highlighted by previously exposed mechanisms as potential glycemic-regulating agents in type 2 diabetes mellitus therapy. These biopeptides could prolong insulin secretion by similar mechanisms to DPP-IV inhibiting drugs such as sitagliptin. Likewise, ACE inhibitors reduce peripheral vascular resistance by downregulating angiotensin II production and bradykinin degradation, therefore sharing similar action mechanisms as ACE-inhibiting drugs. The biological properties of troponin and tropomyosin hydrolysates reported in this study suggest that biopeptides might hold potential health-promoting properties in type 2 diabetes mellitus and hypertension therapy. Nonetheless, determining these nutraceuticals' therapeutic effects is subject to future studies.

The half-maximal concentration of DPP-IV inhibition (EC50) values for the GI-absorbable biopeptides were retrieved to assess the therapeutic potency of the GI-absorbable biopeptides. As shown in Table 2, the therapeutic potency varied significantly among the released peptides. *In vivo* studies demonstrated enhanced insulin sensitivity and postprandial glycemic reduction in diabetic animal models when DPP-IVi peptides were administered (8). Nonetheless, when compared to approved drugs, DDP-IVi biopeptides exhibit a much higher EC50 than DPP-IVi drugs (e.g., sitagliptin [<0.03μM] (35)). Similarly, ACEi peptides show higher EC50 values than ACEi drugs (e.g., captopril [<0.1μM] (36)). These results indicated that biopeptides held a lower therapeutic potency than formerly approved drugs and were not suited to replace current pharmacotherapy. A lower half-life, higher clearance, and lesser affinity for the target protein might be some of the factors underlying the lower therapeutic potency of nutraceuticals. Still, future studies might reveal if biopeptides, concomitantly administered to drugs, could enhance their potency. Currently, evidence in humans is still unknown, partly due to the scarcity of reports regarding the pharmacokinetics of bioactive peptides. Aiming to elucidate this query, drug-likeness compliance and the pharmacokinetic profile of GI-absorbable biopeptides was assessed, and results are presented in Table 3.

Table 3. Biopeptides drug-likeness compliance and pharmacokinetic profile.

Parameter	Bioactive peptide						
	Val-Leu	**Ala-Leu**	**Pro-Leu**	**Ile-Phe**	**Ala-Phe**	**Cys-Phe**	**Gly-Leu**
Drug-likeness compliance (%)							
Lipinski	100.0	100.0	100.0	100.0	100.0	100.00	100.0
Ghose	100.0	100.0	100.0	100.0	100.0	100.	75.0 (LogP<-0.4)[a]
Veber	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Egan	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Muegge	88.9 (LogP<-2)[a]	88.9 (LogP<-2)[a]	88.9 (LogP<-2)[a]	100.0	88.9 (LogP<-2)[a]	88.9 (LogP<-2)[a]	88.9 (LogP<-2)[a]
Absorption							
HIA (≥30%)	High	High	High	High	High	High	High
F (≥30%)	+	+	-	+	+	-	+
Distribution							
PPB (%)	47.4	36.6	46.6	63.9	55.6	53.6	26.8
BBB	+++	+++	++	+++	+++	+++	+++
Metabolism							
CYP450 interactions	None	None	None	None	None	None	None
Excretion							
$T_{1/2}$ (h)	1.4	1.2	0.9	0.7	0.7	0.6	1.3
Clearance ($cm^3 \cdot min^{-1} \cdot kg^{-1}$)	1.3	1.2	1.4	1.5	1.5	1.4	1.0
Toxicity							
DILI	---	---	---	---	---	---	---

HIA, Human Intestinal Absorption; F(≥30%), ≥30% Bioavailability; PPB, Plasma-protein binding; BBB, Blood brain barrier; CYP450, Cytochrome P450; T1/2, half-life; DILI, Drug-Induced Liver Injury. aViolated criteria. (+): probability 0.5-0.7, (++); probability 0.7-0.9, (+++): probability 0.9-1.0, (-): probability 0.3-0.5, (---): probability 0.0-0.1.

Drug-likeness guidelines state several parameters to evaluate the compliance of compounds with physicochemical properties crucial for drug candidates. DPP-IVi bioactive peptides were assessed based on five drug-likeness rules (Lipinski's rule of 5, Ghose filter, Veber's rules, Egan filter, and Muegge filter) which evaluated: molecular weight, molar refractivity, lipophilicity (LogP), topological polar surface area (TPSA), the number of rings, carbons, rotatable bonds, heteroatoms, hydrogen-bond donors and hydrogen-bond acceptors (14). As shown in Table 3, lipophilicity (LogP) was the only violated criterion by all dipeptides except Ile-Phe. The violation of the LogP parameter could be attributed to the amphoteric properties of amino acids. In concordance, Doroshuck *et al.* (37) identified that zwitterions made up of L-amino acids tend to exhibit low LogP values establishing them as highly hydrophilic compounds. Nevertheless, regardless of lipophilicity's slight violation of drug-likeness rules, the lower hydrophobicity could enhance gastrointestinal absorption, bioavailability, and ligand-target interaction (38, 39). While *in silico* evaluation of drug-like properties is limited by the theoretical approach based on topological and molecular descriptors, these parameters determine important chemical features that are expected for a drug candidate when tested *in vivo* (40). Hence, based on the results of this evaluation, bioactive peptides are predicted to exhibit proper drug-like properties when orally administered.

ADME/T analysis was carried out to determine the pharmacokinetics (absorption, distribution, metabolism, excretion, and toxicity) of bioactive peptides. As previously mentioned, seven peptides were filtered according to absorption prediction, and bioavailability was assessed. F(≥30%) value

predicts the probability of achieving a bioavailability greater than 30% when orally administered (41). Five of seven peptides scored >50% chance of surpassing this threshold. Distribution evaluation revealed that GI-absorbable bioactive peptides are likely to cross the blood-brain barrier. Based on the plasma protein binding probability results, peptides are expected to remain in the bloodstream without binding to carrying proteins.

Metabolism was assessed based on the interactions with cytochrome enzymes (CYP450). This large family of oxidoreductase enzymes are tightly involved in xenobiotics metabolism; hence, it is important to predict their interactions with drug-like compounds (42). Bioactive peptides reported no single substrate-like or inhibitory interaction with any CYP450. On the other hand, when assessing toxicity, results show that peptides induce no liver injury based on the DILI parameter. Our findings support the hypothesis of Acquah *et al.* (8) since *in silico* evaluation indicated that biopeptides exert little complications in humans in terms of toxicity and organ damage. At last, excretion was evaluated based on half-life and clearance rate. Half-life varied irregularly among these peptides, and only three reported a value higher than 1 hour. On the other hand, clearance predicted values were all above 1 $cm^3 \cdot min^{-1} \cdot kg^{-1}$. These two parameters must be carefully considered, as low half-life and high clearance could imply an increased dosing regimen and consumption frequency to achieve an effective therapeutic effect (43). This work develops a non-previously analyzed pharmacokinetic profile of bioactive peptides, yet, in vivo studies are still imperative to conclude with a greater degree of certainty.

Target Prediction

Bioactive peptides exhibit a doubtless affinity for a broad spectrum of proteins (44). Hence, addressing the possible target for these ligands is crucial when assessing the bioactive potential of food-hydrolysates. Results from the three proteins with the highest binding affinity with each of the biopeptides are presented in Table 4.

Table 4. Predicted target proteins for GI-absorbable biopeptides.

Biopeptide Sequence	Target proteins (Probability[a])		
	Protein 1	Protein 2	Protein 3
Val-Leu	Calpain-I (0.25)	ACE (0.15)	COX-2 (0.13)
Ala-Leu	COX-2 (0.21)	DPP-IV (0.09)	HLA-A3 (0.08)
Pro-Leu	DPP-IV (0.42)	ACE (0.28)	Calpain-I (0.19)
Ile-Phe	Calpain-I (0.54)	ACE (0.50)	COX-2 (0.17)
Ala-Phe	Calpain-I (0.30)	Neprilysin (0.16)	SMOT (0.16)
Cys-Phe	Calpain-I (0.08)	ACE (0.06)	Neprilysin (0.06)
Gly-Leu	COX-2 (0.13)	HLA-A3 (0.10)	ACE (0.09)

ACE, Angiotensin Converting Enzyme; COX-2, Cyclooxygenase-2; DPP-IV, Dipeptidyl-Peptidase IV; SMOT, Small Intestine Oligopeptide Transporter; HLA-A3, Human Leukocyte Antigen-A3. [a]Probability of the biopeptide to have each protein as a target.

Based on the results obtained from *in silico* target prediction analysis, seven suitable proteins were reported to be likely bonded to GI-absorbable bioactive peptides. Five of seven biopeptides exhibited a binding affinity for ACE in line with their anti-hypertensive properties. Furthermore, possible interactions with neprilysin were reported for peptides Ala-Phe and Cys-Phe. Neprilysin is a Zn^{2+}-dependent protease tightly involved in the renin-angiotensin-aldosterone system, particularly catalyzing natriuretic peptides degradation (45). Sacubitril (the first neprilysin inhibitor approved) has shown promising results for hypertension therapy when administered concomitantly with angiotensin-receptor blockers (46). Based on the findings of neprilysin affinity, the results suggest that biopeptides could modulate vascular resistance through ACE inhibition and this protease. Further studies could elucidate these interactions' feasibility and therapeutic application.

Interestingly, a high probability of interaction with cyclooxygenase-2 (COX-2) was also reported.

COX-2 is a rate-limiting enzyme that catalyzes the production of prostaglandins. Hence, its inhibition conveys a crucial anti-inflammatory mechanism shared by non-steroidal anti-inflammatory drugs (NSAIDs). Several COX-2 inhibitors of natural origin have been identified, as stated by Cui *et al.* (47), yet bioactive peptides have not been reported among these naturally occurring anti-inflammatory compounds. Our results and findings of Reyes-Díaz *et al.* (48) (who isolated anti-inflammatory biopeptides from legume proteins) suggest that bioactive peptides derived from dietary have anti-inflammatory properties. Therefore, we encourage a study of those peptides *in vitro* and *in vivo*. Moreover, human leukocyte antigen-A3 (HLA-A3) is classically associated with hemochromatosis, but it has also been linked to antiviral T cell-mediated immune responses (49). Biopeptides Ala-Leu and Gly-Leu exhibit affinity to HLA-A3, yet the nature of these interactions remains to be unrevealed. The small intestine oligopeptide transporter (SMOT) regulates the peptide absorption from dietary intake (50) and was reported to be bonded to the dipeptide Ala-Phe; however, little is known about the effect of bioactive peptides on the modulation (if not only a diffusion-channel into enterocytes) of SMOT.

Regarding the anti-diabetic properties of bioactive peptides, DPP-IV inhibition indicates a high potential as a glycemic-regulatory agent. Additionally, Calpain-I was identified as a suitable target protein for five biopeptides, and four showed the highest affinity for this enzyme. Calpain-I, a Ca^{2+}-dependent cytosolic protease, is pathologically triggered by alterations in calcium homeostasis commonly seen in diabetes mellitus (51). These proteases have been involved in the pathogenesis of several complications, including diabetic cardiomyopathy (via activation of the nuclear factor of activated T-cells (NFAT) and nuclear factor-κB (NF-κB) which seems to lead to ventricular fibrosis (52)), diabetic nephropathy (associated with cellular death and renal dysfunction (53)), and diabetic retinopathy by induced-proteolysis (causes early degeneration of cytoskeleton and structure involved in phototransduction in retinal cells (51)). As a result, modulators/inhibitors of Calpain-I activity are proposed as potential therapeutic agents for type 2 diabetes mellitus (54, 55). Henceforth, results derived from this investigation indicate that bioactive peptides could exert a health-promoting effect in diabetes mellitus aside from potentiating the incretin effect.

CONCLUSION

Troponin and tropomyosin are rich dietary sources of bioactive peptides, mainly DPP-IV and ACE inhibitors. The hydrolysis of these myofibrillar proteins shows no significant difference ($p<0.05$) between the frequency of biopeptides released from the isoforms of *B. taurus*, *G. gallus*, and *S. scrofa*. Eight biopeptides were obtained from simulated gastric and pancreatic digestion of troponin and tropomyosin: DPP-IV inhibitors, ACE inhibitors, CaMPDE inhibitors, renin inhibitors, DPP-III inhibitors, glucose-uptake stimulating, ion-flux regulating, and antioxidants.

Seven biopeptides from the whole end-products were classified as absorbable compounds and exhibited appropriate drug-like properties. The pharmacokinetic profiling revealed a predicted high blood-brain barrier diffusion, no CYP450 interaction, and null toxicity. Even with the pharmacological properties, the therapeutic potency of biopeptides is lower than those drugs, indicating that the nutraceuticals reported in this research must not replace the current pharmacotherapy. The target prediction suggests that biopeptides could: [1] regulate glycemia not only by interacting with DPP-IV but also with Calpain-I, [2] exert antihypertensive action by binding to ACE and neprilysin, and [3] hold anti-inflammatory properties as they reported high affinity for COX-2. These interactions remain to be elucidated in future in vitro and in vivo studies.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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