Biointerface Research in Applied Chemistry
Platinum Open Access Journal (ISSN: 2069-5837)

Article
Volume 12, Issue 4, 2022, 4929 - 4944
https://doi.org/10.33263/BRIAC124.49294944

Discovery of Bifunctional Anti-DPP-IV and Anti-ACE Peptides from Housefly Larval Proteins After *In silico* Gastrointestinal Digestion

Jiun-An Koh [1], Joe-Hui Ong [1], Fazilah Abd Manan [2], Kah-Yaw Ee [1,3], Fai-Chu Wong [1,3], Tsun-Thai Chai [1,3,*]

[1] Faculty of Science, Universiti Tunku Abdul Rahman, 31900 Kampar, Perak, Malaysia; jiunan30@1utar.my (J.-A. K.), candy3997@gmail.com (J.-H. O.), eeky@utar.edu.my (K.-Y. E.), wongfc@utar.edu.my (F.-C. W), chaitt@utar.edu.my (T.-T. C.);
[2] Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia; m-fazilah@utm.my (F.A.M.);
[3] Center for Agriculture and Food Research, Universiti Tunku Abdul Rahman, 31900 Kampar, Perak, Malaysia
* Correspondence: chaitt@utar.edu.my (T.-T.C.);

Scopus Author ID 36150542400
Received: 6.08.2021; Revised: 12.08.2021; Accepted: 18.08.2021; Published: 17.10.2021

Abstract: Proteins and peptides of housefly larvae (HFL) have potential applications in food and therapy. The fate of HFL proteins following human gastrointestinal (GI) digestion is unknown. This study adopted a computational approach to discover peptides released from HFL proteins upon GI digestion. *In silico* digestion of eight major HFL proteins released 783 peptides. This comprised 243 peptides exhibiting 13 types of bioactivities. Ninety-two single-function peptides exhibiting anti-dipeptidyl peptidase IV (anti-DPP-IV), anti-dipeptidyl peptidase III, anti-angiotensin converting enzyme (anti-ACE), or antioxidant activity were found. Sixty-three multi-function peptides, encompassing 32 bifunctional anti-DPP-IV and anti-ACE peptides, were found. Further screening led to five non-toxic, non-allergenic, high-GI-absorption bifunctional dipeptides: AF, GW, GY, PH, and VF. Molecular docking found the dipeptides to interact with the active site of DPP-IV through hydrophobic interactions. Only GW and VF could bind to the active site of ACE. Thus, the five dipeptides are competitive inhibitors of DPP-IV. GW and VF are potential competitive inhibitors of ACE, whereas AF, GY, and PH are non-competitive inhibitors. Overall, GI digestion could liberate numerous single- and multi-function peptides from HFL proteins. Hence, HFL proteins can be tapped for potential applications in antidiabetic and antihypertension functional food and therapy.

Keywords: bioactive peptide, *in silico*, bioinformatics, housefly larva, dipeptidyl peptidase IV, angiotensin-converting enzyme, BIOPEP

1. Introduction

Bioactive peptides are short peptides encrypted within the structure of a parental protein, which exert biologically relevant activities following their liberation. At present, bioactive peptides which exert anticancer, antidiabetic, antioxidant, antiviral, and many other bioactivities have been documented in the literature [1, 2]. Bioactive peptides can be generated by enzymatic hydrolysis, which could happen *in vivo* during the gastrointestinal (GI) digestion of ingested dietary proteins, or through food processing technology, such as microbial fermentation of protein-rich foods [3]. The past decade has seen a surge in researchers' interest

in discovering bioactive peptides from diverse biological samples. Bioactive peptides have been identified from edible and non-edible animals [4–6] and plant [7–9] sources. One factor fueling such enthusiasm is the recognition of the potential applications of bioactive peptides as functional food ingredients and therapeutic/prophylactic agents [2, 3].

To date, bioactive peptide discovery is largely driven by wet-lab experimentations that involve the release of bioactive peptides from protein sources, bioactivity-guided purification, mass spectrometric identification of peptide sequences, synthesis of peptides, and validation of bioactivity [3, 10]. Often, the identification of a few single-function bioactive peptides would be accomplished by such a strategy. Notwithstanding, it is increasingly evident that bioactive peptides can be multifunctional. For instance, three multifunctional peptides exhibiting antibacterial, anti-angiotensin converting enzyme (anti-ACE) and anti-dipeptidyl peptidase IV (anti-DPP-IV) activities have been identified from fish gelatin hydrolysates [11]. In addition, three bifunctional peptides exhibiting anti-ACE and antioxidant activities were identified from egg white hydrolysate [12]. Meanwhile, 10 bifunctional peptides with anti-DPP-IV and anti-ACE activities *in vitro* were identified from egg white ovalbumin [13]. Multifunctional peptides are preferable to single-function peptides. The formers are potentially more versatile, allowing the modulation of multiple *in vivo* pathways and thus imparting multiple health benefits [14]. Such multifunctionality is particularly relevant in the context of relatively complex disorders. An example would be that patients with type II diabetes also often experience hypertension [15]. Zhang *et al*. [12] proposed that in light of the multifactorial nature of the pathogenesis of hypertension, a bifunctional peptide with concurrent anti-ACE and antioxidant effects may have a better therapeutic effect than a single-function anti-ACE peptide.

Housefly larvae (HFL) have emerged as a potential source of bioactive peptides and proteins only in recent years. By contrast, their significance as a sustainable and alternative source of proteins for animal feed production is well-recognized. Generally, proteins of HFL and other insects are regarded as a promising solution to the increasing global demand for proteins for human food and animal feed production. Crickets and mealworms, for example, are already being used commercially in human food production. By contrast, at present, HFL is mainly used for animal feed production [16–18]. Notably, several studies have pointed to the health-promoting potential of HFL proteins and peptides. For example, two studies have reported the antioxidant activities of HFL protein hydrolysates [19, 20]. A protein-rich HFL extract was found to exert liver- and pancreas-protective activities in the type 2 diabetic rat model, giving no signs of sub-chronic toxicity [21]. Endothelial dysfunction is a key factor in vascular disease. In relation to this, HFL polypeptide extract inhibited the dysfunction of human umbilical vein endothelial cells when challenged by inflammatory cells [22].

The current understanding of the health benefits of HFL peptides in humans is far from comprehensive. Bioinformatic or *in silico* research strategies can be adopted to expedite the exploration of such information. *In silico* tools encompassing peptide databases, bioactivity prediction servers, and molecular docking software have been frequently used in bioactive peptide research in recent years. The BIOPEP-UWM database [23], for example, has been used in some studies to screen for potential protease treatments for the generation of bioactive protein hydrolysates. It has also been used to predict and compare the bioactive peptides that could be generated from dietary proteins [24, 25]. While molecular docking tools have been adopted in docking-based screening for bioactive peptides in some studies, others have also used the tools to elucidate the mechanisms of intermolecular interactions between bioactive

peptides and their protein targets [26, 27]. Generally, the cost- and time-effectiveness of the *in silico* approach has driven their popularity in bioactive peptide research [28]. At present, the number of bioactive peptides identified from HFL proteins is still limited. The fate of HFL proteins following human GI digestion is also unknown. Thus, in this study, we have adopted an *in silico* approach to unravel multifunctional bioactive peptides that may be generated from HFL proteins following *in silico* GI digestion. The discovery of such peptides, although theoretical, could offer preliminary evidence, serving as the foundation for future work to investigate the health benefits of HFL-derived peptides in the context of the application of HFL proteins for human consumption.

2. Materials and Methods

2.1. In silico GI Digestion.

Figure 1 shows the overall strategy adopted in this computational study to discover potential multifunctional peptides from the major HFL proteins following degradation by GI proteases. The sequences of the eight major proteins, as previously reported [29], were downloaded from the UniProtKB database (https://www.uniprot.org/) [30] (access date: 21 May 2021). *In silico* GI digestion of the eight proteins was performed by using the "enzyme(s) action" tool on the BIOPEP-UWM server (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) [23] (access date: 22-24 May 2021). GI digestion was simulated using pepsin, trypsin, and chymotrypsin A, as previously described [27]. All bioactive peptides resulting from the *in silico* GI digestion were recorded. The peptides were subsequently divided into different single- and multi-function groups.

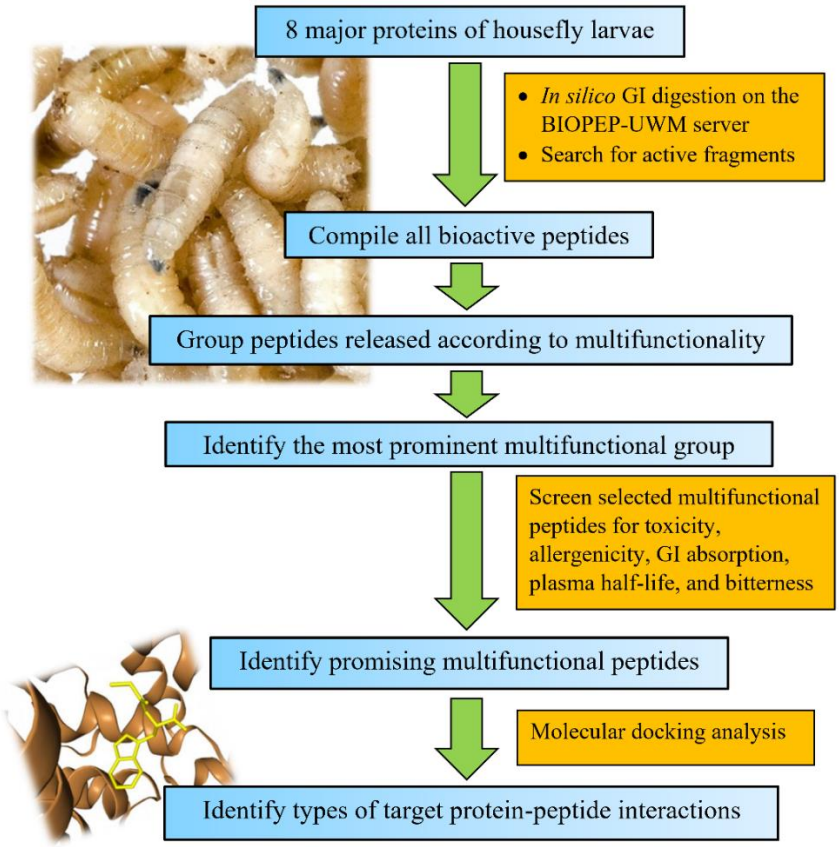

Figure 1. Overview of the study.

2.2. Screening for toxicity, allergenicity, GI absorption, plasma half-life, and bitterness.

The multifunctional group with the largest number of peptides was screened for toxicity with ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/index.html) [31]. The resultant non-toxic peptides were screened for allergenicity using AllerTOP v.2.0 (https://www.ddg-pharmfac.net/AllerTOP/) [32]. Peptide sequences predicted as "Non-toxin" and "Probable Non-Allergen" were converted into the Simplified Molecular Input Line Entry System (SMILES) format by using the "SMILES" tool on BIOPEP-UWM [23] and then submitted to SwissADME (http://www.swissadme.ch/) [33] to predict for GI absorption. The plasma half-life of the peptides was predicted by using the PlifePred web server (http://webs.iiitd.edu.in//raghava/plifepred/) [34]. Bitterness was predicted by using the "profiles of sensory activity" tool on BIOPEP-UWM [35]. The aforementioned web tools were accessed between 25 and 30 May 2021.

2.3. Molecular docking analysis.

The docking of selected peptides onto two target proteins was carried out with HPEPDOCK (http://huanglab.phys.hust.edu.cn/hpepdock/) [36] (access date: 2 June 2021). Two crystal structures from the RCSB Protein Data Bank (https://www.rcsb.org/) [37], namely human ACE complexed with bradykinin potentiating peptide b (PDB ID: 4APJ) [38] and human DPP-IV complexed with diprotin A (PDB ID: 1WCY) [39], were used. The docking of peptides onto ACE and DPP-IV was performed by submitting the peptide sequences in the FASTA format as peptide input. The top (most negative) docking scores for the peptides were tabulated. BIOVIA Discovery Studio Visualizer (BIOVIA, Dassault Systèmes, BIOVIA Discovery Studio Visualizer, Version 20.1.0.192, San Diego: Dassault Systèmes, 2020) and ProteinsPlus web service (https://proteins.plus) [40, 41] were used for the visualization of the 3D structures of docked models. Intermolecular interactions between a peptide and a target protein were visualized and analyzed using LigPlot+ v.2.2 [42, 43].

3. Results and Discussion

In silico GI digestion of eight major HFL proteins generated a total of 783 peptide fragments (Table 1), ranging from 2 to 18 residues (data not shown). Generally, more peptides were released from the proteins of greater molecular masses and peptide length. In this study, catalase (56.7 kDa) released the largest number of peptides (25%), about 4.5-fold more than those released from superoxide dismutase [Cu-Zn] (15.6 kDa). This observation reflects a greater number of pepsin, trypsin, and chymotrypsin cleavage sites in the protein sequence of catalase than in superoxide dismutase [Cu-Zn]. Our observation also agrees with a previous report that patatins, which are about 1.8-fold larger than sporamins in mass, released about 2-fold more peptide fragments than did sporamins, when both were digested *in silico* on the BIOPEP-UWM server [44].

Table 1. Major HFL proteins and the number of peptides released from them by *in silico* GI digestion.

Protein	UniProt ID	Number of residues	Molecular mass (kDa)	Number of peptides released
Catalase	T1PCG9	505	56.7	197
Glutathione reductase family member	P91884	495	53.4	166
Superoxide dismutase	G3GJ67	214	24.2	92
Ferritin	T1PLJ3	205	23.1	85
AhpC/TSA family	T1PEX1	220	24.9	82
Antifungal peptide-1	G9B2K0	193	21.2	72

Protein	UniProt ID	Number of residues	Molecular mass (kDa)	Number of peptides released
Cytochrome c	T1PF88	108	11.7	45
Superoxide dismutase [Cu-Zn]	Q6SCL6	153	15.6	44
Total				783

Our analysis of the 783 peptides (Table 1) by using the BIOPEP-UWM server resulted in a collection of 243 peptides exhibiting 13 types of bioactivities (Table 2). Most of the bioactive peptides (94%) are dipeptides (data not shown). Each of the eight HFL proteins could release anti-DPP-IV, anti-ACE, and antioxidant peptides. By contrast, peptides with anti-amnestic and hypolipidemic activities and those capable of stimulating the release of vasoactive substances were rare; only one peptide sequence was detected for each bioactivity type. Overall, 47% of the bioactive peptides detected were anti-DPP-IV peptides, whereas 33% were anti-ACE peptides. In line with our findings, *in silico* proteolysis of fish-roe-derived vitellogenin on the BIOPEP-UWM server revealed that anti-DPP-IV and anti-ACE peptides comprised most of the bioactive peptides detected [45]. Furthermore, *in silico* investigation on nine tomato seed proteins also found drastically greater frequencies of anti-DPP-IV and anti-ACE peptides compared to antioxidant peptides [46]. Our observations and those of others could be attributed to the enrichment of the BIOPEP-UWM database with anti-DPP-IV and anti-ACE peptides. Among the 4325 peptides deposited in the database, about 427 and 1051 of them were anti-DPP-IV and anti-ACE peptides, respectively (access date: 03 July 2021) [23]. In this study, catalase stood out from the rest as it released the largest number of bioactive peptides (28%), following *in silico* GI digestion (Table 2). Thus, future research could focus on the catalase protein by adopting a targeted approach to discover bioactive peptides from selected HFL proteins following GI digestion or other proteolytic treatments.

Table 2. Bioactive peptides released from HFL proteins by *in silico* GI digestion.

	Protein								
Bioactivity	Catalase	Glutathione reductase family member	AhpC/TSA family	Superoxide dismutase	Antifungal peptide-1	Superoxide dismutase [Cu-Zn]	Cytochrome c	Ferritin	**Total number**
Anti-DPP-IV	32	23	15	16	10	9	5	5	115
Anti-ACE	20	22	9	6	9	6	7	2	81
Antioxidant	4	1	2	2	1	1	1	2	14
Anti-DPP-III	5	3	2	0	0	1	0	0	11
Anti-renin	4	0	0	0	0	1	0	0	5
Stimulating glucose uptake	2	2	0	0	0	0	0	0	4
Regulating ion flow	0	3	0	1	0	0	0	0	4
Anti-alpha-glucosidase	1	0	0	0	1	0	0	0	2
Anti-CaMPDE[a]	1	0	0	0	0	1	0	0	2
Anti-inflammatory	0	0	1	1	0	0	0	0	2
Anti-amnestic	0	0	0	1	0	0	0	0	1
Hypolipidemic	0	0	0	0	0	1	0	0	1
Stimulating vasoactive substance release	0	0	0	1	0	0	0	0	1
Number of bioactive peptides released	69	54	29	28	21	20	13	9	243

[a] CaMPDE, calmodulin-dependent phosphodiesterase 1.

To facilitate the discovery of multifunctional peptides, the bioactive peptides reported in Table 2 were further divided into monofunctional peptides and multifunctional peptides, as

shown in Table 3. A total set of 92 monofunctional peptides exhibiting anti-DPP-IV, anti-ACE, antioxidant or anti-DPP-III activities was detected. The other nine types of bioactivities were found only in multifunctional peptides. Among the 115 anti-DPP-IV peptides (Table 2), 56 were found as multifunctional peptides, concurrently exhibiting anti-DPP-IV and at least one other bioactivity (Table 3). The 32 bifunctional peptides with anti-DPP-IV and anti-ACE activities are the most prominent among the multifunctional groups. The bifunctional anti-DPP-IV + anti-ACE peptides consist of only dipeptides (data not shown). Unlike the anti-DPP-IV + anti-ACE peptides, not more than 5 peptide sequences were detected for the other multifunctional groups. Thus, our subsequent *in silico* and molecular docking analyses focused on the bifunctional anti-DPP-IV + anti-ACE peptides.

Table 3. Single- and multi-function bioactive peptides released from HFL proteins by *in silico* GI digestion.

Type of bioactive peptides	Bioactivity	Number of peptides
Monofunctional	Anti-DPP-IV	59
	Anti-ACE	26
	Antioxidant	6
	Anti-DPP-III	1
Bifunctional	Anti-DPP-IV + anti-ACE	32
	Anti-ACE + regulating ion flow	4
	Anti-DPP-IV + glucose uptake stimulating	3
	Anti-DPP-IV + anti-DPP-III	2
	Anti-DPP-IV + anti-inflammatory	2
	Anti-ACE + antioxidant	1
	Anti-ACE + anti-DPP-III	1
Trifunctional	Anti-DPP-IV + anti-ACE + anti-DPP-III	5
	Anti-DPP-IV + anti-ACE + antioxidant	4
	Anti-DPP-IV + anti-ACE + anti-renin	1
	Anti-DPP-IV + anti-ACE + stimulating glucose uptake	1
	Anti-DPP-IV + stimulating vasoactive substance release + anti-amnestic	1
Tetrafunctional	Anti-DPP-IV + anti-ACE + antioxidant + anti-alpha-glucosidase	2
	Anti-DPP-IV + anti-ACE + anti-DPP-III + anti-renin	2
	Anti-ACE + anti-renin + anti-CaMPDE + hypolipidemic	1
Pentafunctional	Anti-DPP-IV + anti-ACE + antioxidant + anti-renin + anti-CaMPDE[a]	1

[a] CaMPDE, calmodulin-dependent phosphodiesterase 1.

Screening of the 32 anti-DPP-IV + anti-ACE dipeptides (Table 3) revealed five that were predicted to possess desirable qualities, namely non-toxic, non-allergenic, and high GI absorption (Table 4). The five dipeptides are not unique to HFL, but also released by other dietary proteins following proteolysis. For example, GY and VF were also released by *in silico* GI digestion of oat globulins [47]. PH was identified from anti-ACE milk hydrolysates [48], whereas VF is a competitive anti-ACE inhibitor identified from a sardine muscle hydrolysate [49]. GW, GY, and AF were anti-ACE peptides identified from fermented soybean seasoning, which was shown to have antihypertensive effects in animal models [50].

Table 4. Toxicity, allergenicity, GI absorption, plasma half-life, and bitterness predicted five bifunctional anti-DPP-IV and anti-ACE peptides.

Peptide	Toxicity	Allergenicity	GI absorption	Plasma half-life (seconds)	Bitterness
AF	Non-toxin	Probable non-allergen	High	834.81	Bitter
GW	Non-toxin	Probable non-allergen	High	834.81	Non-bitter
GY	Non-toxin	Probable non-allergen	High	834.71	Bitter
PH	Non-toxin	Probable non-allergen	High	834.81	Non-bitter
VF	Non-toxin	Probable non-allergen	High	834.81	Bitter

GW and VF were also purified from katsuobushi and validated for anti-DPP-IV activities *in vitro* [51].

Gupta, *et al*. [31] suggested that non-toxin peptides usually contain valine, threonine, arginine, glutamine, methionine, leucine, lysine, isoleucine, phenylalanine, and alanine. This description matches the dipeptides AF and VF in this study. Besides toxicity, allergenicity should also be considered when evaluating the potential of bioactive peptides as future food ingredients or therapeutic agents. This ensures that the peptides could exert their health-promoting or disease-preventing effects when consumed, without causing undesirable immunological responses or harmful side effects [32]. Furthermore, bioactive peptides proposed for applications as food ingredients or therapeutic agents should have high bioavailability. Bioactive peptides can only modify physiological pathways if they can reach the *in vivo* targets after being absorbed through the intestinal wall [52]. In this context, the five bifunctional peptides in Table 4 are promising as they were predicted to show high GI absorption, in addition to being resistant to degradation by GI enzymes. The latter is implicated as the five dipeptides were produced following *in silico* digestion by GI proteases. The uptake of dipeptides into the mammalian small-intestinal enterocytes has been well-established in the literature, which is facilitated by a PepT1 H^+/peptide co-transporter. The co-transporter is known to have a very broad substrate specificity encompassing about 400 dipeptides [53]. Thus, the high GI absorption predicted for the five bifunctional peptides in this study is anticipated.

Among other factors, the expression of *in vivo* bioactivity requires a peptide to be stable in blood [34, 54]. In this study, the five bifunctional dipeptides in Table 4 did not differ in their predicted plasma half-life values. This implies that they are likely similarly susceptible to plasma peptidases during systemic circulation. Mathur *et al*. [34] reported that peptides enriched in aromatic and neutral residues are more likely to have a shorter half-life than other peptides; the five bifunctional dipeptides we found in this study comprise those amino acids. PlifePred predicted that glutathione, a natural tripeptide, had a plasma half-life about 9-fold longer than the five bifunctional dipeptides (data not shown). The uncommon first peptide linkage could account for this in glutathione that involves a gamma-carboxyl group in its formation. In fact, structural modification strategies, such as cyclization and N- or C-terminal acetylation/amidation, have been proposed to enhance the *in vivo* half-life of bioactive peptides [54]. Such strategies would be useful in the future to improve the bioavailability of the five bifunctional dipeptides.

AF, GY, and VF were predicted to be bitter (Table 4). As reported by Iwaniak *et al*. [55], bitterness is associated with the presence of branched-chain amino acids (such as valine) and aromatic amino acids (such as phenylalanine and tyrosine) in the composition of a peptide. The significance of phenylalanine and tyrosine in imparting bitterness could be attributed to their ability to bind to the bitter taste receptors of the gustation cells [56]. In general, although the five bifunctional peptides could be potentially developed into dual-function anti-DPP-IV and anti-ACE therapeutics in the future, the non-bitter GW and PH would be preferable when considering the formulation of functional food from these five peptides. The integration of non-bitter peptides would be less likely to adversely affect the taste profile of orally-taken products, hence more likely to be acceptable to consumers.

To understand the mechanisms of the five bifunctional peptides as DPP-IV and ACE inhibitors, we performed molecular docking analyses on the peptides. The empirical evidence of the anti-DPP-IV and anti-ACE activities of the five peptides was reported [48–50, 57, 58].

However, to the best of our knowledge, systematic docking analysis of the five dipeptides on DPP-IV and ACE has not been reported in the same study. Molecular docking of VF and AF on ACE [59, 60] and that of GW on DPP-IV [61] was reported. However, except for VF, details on the interactions of the five bifunctional peptides with ACE and DPP-IV are still unavailable.

The active site of DPP-IV comprises the catalytic triad (Ser630, Asn710, and His740), the hydrophobic S1 pocket (Tyr631, Val656, Trp659, Tyr662, Tyr666, Val711), and the S2 pocket (Arg125, Glu205, Glu206, Ser209, Phe357, Arg358) [62]. Our molecular docking simulations revealed that the five bifunctional dipeptides could bind to the catalytic triad and the two pockets mainly through hydrogen bonds and hydrophobic interactions (Table 5). GW was predicted to have the most negative docking scores among the five dipeptides, implying the most stable binding to DPP-IV [63]. A graphical representation of the intermolecular interactions between GW and DPP-IV is shown in Figure 2.

Table 5. Docking scores of bifunctional anti-DPP-IV and anti-ACE peptides and their intermolecular interactions with DPP-IV.

		Docking score	Interaction with residues of DPP-IV[a]	
			Hydrogen bond	Hydrophobic interaction
Bifunctional anti-DPP-IV + anti-ACE peptides	GW	-141.824	Arg125(2), Glu205, Glu206	Arg125, Glu205, Glu206, Tyr547, **Ser630**, Tyr631, Val656, Trp659, Tyr662, Tyr666, **Asn710**, Val711, **His740**
	PH	-132.725	Arg125, Tyr662	Arg125, Glu205, Glu206, Tyr547, **Ser630**, Tyr631, Val656, Trp659, Tyr662, Tyr666, **His740**
	VF	-128.224	Arg125(2), Glu205, Glu206, **His740**	Arg125, Glu205, Glu206, Tyr547, **Ser630**, Tyr631, Val656, Trp659, Tyr662, Tyr666, **Asn710**, Val711, **His740**
	GY	-122.504	Tyr662	Arg125, Glu205, Glu206, Tyr547, **Ser630**, Tyr631, Val656, Trp659, Tyr662, Tyr666, **Asn710**, Val711, **His740**
	AF	-112.008	Arg125, Tyr547, **His740**	Arg125, Glu205, Glu206, Tyr547, **Ser630**, Tyr631, Val656, Trp659, Tyr662, Tyr666, **Asn710**, Val711, **His740**
Reference peptides	CF	-115.889	Arg125(2), Glu206	Arg125, Glu205, Glu206, Phe357, Tyr547, **Ser630**, Tyr631, Val656, Tyr662, Tyr666, Val711, **His740**
	KM	-103.657	Arg125, Glu205, Glu206, Ser209	Arg125, Glu205, Glu206, Ser209, Tyr547, **Ser630**, Tyr631, Val656, Tyr662, Tyr666, **Asn710**, Val711, **His740**
	AM	-93.654	Arg125, Glu206	Arg125, Glu205, Glu206, Tyr547, **Ser630**, Tyr631, Val656, Tyr662, Tyr666, **Asn710**, Val711, **His740**

[a] Number in brackets indicates the number of hydrogen bonds formed with the same DPP-IV residue. Residues in **bold** indicate catalytic triad residues of DPP-IV. Other residues in the active site of DPP-IV are underlined.

As shown in Table 5, except for PH, the bifunctional peptides were predicted to bind to all three residues in the catalytic triad by hydrophobic interactions. VF and AF could also bind to His740 of the catalytic triad by means of the hydrogen bond. Our result suggests that hydrogen bonds play a greater role in binding the peptides to the S2 pocket than to the S1 pocket of DPP-IV. Only GY and PH could hydrogen-bond to residue in the S1 pocket (Tyr662). By contrast, except for GY, the bifunctional peptides could hydrogen-bond to at least one of the residues in the S2 pocket, mainly with Arg125, Glu205, and Glu206. This is desirable as binding to Glu205 and Glu206 in the S2 pockets is believed to play an important role in stabilizing the binding of inhibitors to DPP-IV [64].

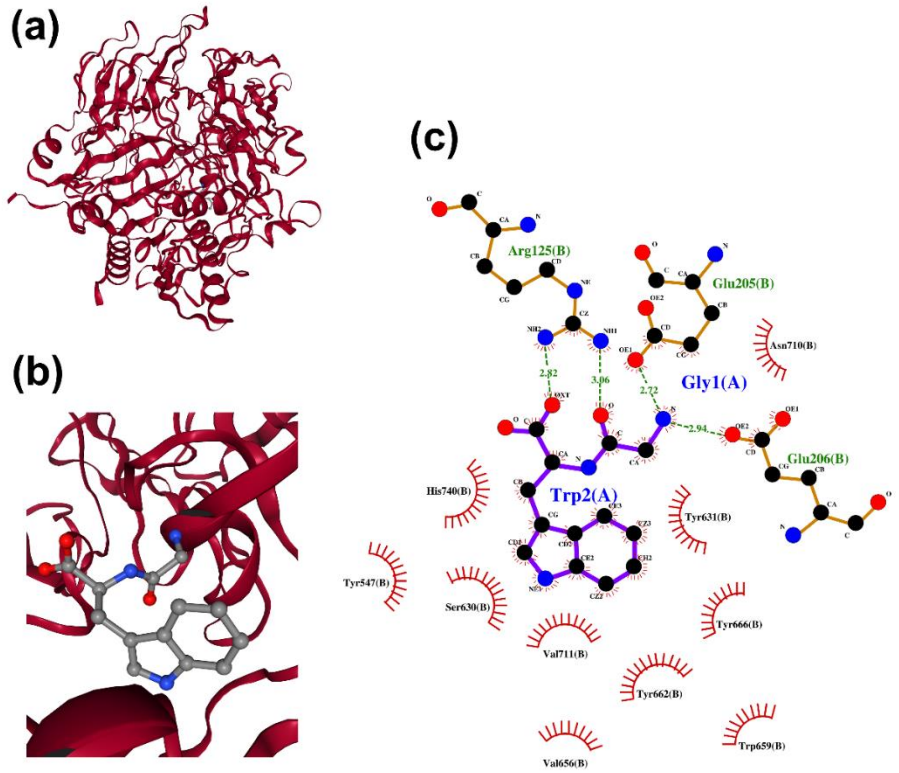

Figure 2. The GW-DPP-IV docked model presented in 3D (**A**, **B**) and 2D (**C**) diagrams. The protein is shown in the cartoon in maroon; the peptide is presented in a ball-and-stick style. In the 2D diagram (**C**), the hydrophobic bonds and hydrogen bonds are displayed in red spoked arcs, and green dashed lines, respectively.

GW has a more negative docking score than VF, suggesting that GW may form a more stable binding to DPP-IV compared with VF. The greater binding stability of GW to DPP-IV corroborates with the report of the stronger *in vitro* anti-DPP-IV activity of GW, relative to VF [51]. Meanwhile, VF could form almost the same interactions with DPP-IV as could GW, except for one additional hydrogen bond (with catalytic residue His740) involving VF, but not GW (Table 5). Our results imply that the additional hydrogen bond with a catalytic residue would neither contribute to greater binding stability between a dipeptide and DPP-IV, nor enhance the anti-DPP-IV activity of a dipeptide. A logical hypothesis, in this case, is that the specific identity of the peptide residue forming a hydrogen bond and/or hydrophobic interaction with DPP-IV would influence the contribution of that interaction to the overall docking stability.

CF, KM, and AM are three reference dipeptides we adopted, whose anti-DPP-IV and anti-ACE activities have been verified empirically [13]. Our LigPlot analysis revealed that GW and VF resemble KM in that all three of them could interact with Arg125, Glu205, and Glu206 in the S2 pocket via hydrogen bonds and hydrophobic interactions. Furthermore, the three of them can form hydrophobic interactions with the catalytic triad of DPP-IV. Based on the comparison of docking scores, four HFL-derived dipeptides (GW, PH, VF, and GY) apparently could bind to DPP-IV more stably than the three reference peptides. However, at this point, it is uncertain whether the four HFL peptides would be more potent than the three reference peptides. GW, which could bind more stably to DPP-IV relative to VF in this study, was previously reported to have greater anti-DPP-IV activity than VF [51]. However, the binding stability of the three reference peptides in this study does not correlate with the report that their relative anti-DPP-IV activities, in descending order, are KM > AM > CF [13]. In short, binding stability is apparently not the main factor determining the anti-DPP-IV activity of all

dipeptides. In the case of the three reference peptides we adopted, the ability of KM and AM to bind to all three residues of the catalytic triad of DPP-IV might have contributed to their stronger activities relative to CF.

The active site of ACE comprises the inhibitor binding site (His383, His387, and Glu411), S1 pocket (Ala354, Glu384, and Tyr523), S2 pocket (Gln281, His353, Lys511, His513, and Tyr520), and S1' (Glu162) [65]. Unlike our results for docking on DPP-IV, only two bifunctional dipeptides GW and VF, could bind to residues in the active site pockets (Table 6). GW and VF were both predicted to hydrogen-bond to residues in the S2 pocket. By means of hydrophobic interactions, both GW and VF could bind to S1 and S2 pockets, but only VF could bind to the S1' pocket. As in docking to DPP-IV (Table 5), the docking of GW to ACE also produced the most negative score, indicating the most stable binding to the active site pockets. Notably, GW is the only bifunctional dipeptide that was predicted to bind to all three residues in the ACE inhibitor binding site. Figure 3 shows GW binding to the active site of ACE through hydrogen bonds and hydrophobic interactions.

Table 6. Docking scores of bifunctional anti-DPP-IV and anti-ACE peptides and their intermolecular interactions with ACE.

		Docking score	Interaction with residues of ACE[a]		
			Hydrogen bond	Hydrophobic interaction	Salt bridge
Bifunctional anti-DPP-IV + anti-ACE peptides	GW	-117.560	Gln281, Asp415, Asp453	Gln281, **His383**, **His387**, **Glu411**, Asp415, Phe457, His513, Tyr520, Tyr523, Phe527	-
	PH	-116.371	Asp358, Tyr360	Ala356, Trp357, Asp358, Tyr360, Phe391, Tyr394, Arg402, Glu403, His410	Glu403
	VF	-108.293	Lys511(2), Tyr520	Tyr146, Glu162, Trp279, Gln281, His353, Phe457, Lys511, Phe512, His513, Tyr520, Tyr523, Phe527	-
	GY	-97.373	Glu123	Trp59, Tyr62, Ile88, Glu123, Arg124, Tyr360	-
	AF	-94.634	-	Trp59, Tyr62, Ile88, Glu123, Arg124, Tyr360	-
Reference peptides	CF	-108.306	-	Trp59, Tyr62, Ile88, Leu122, Glu123, Arg124, Ala125, Tyr360	-
	KM	-93.624	Glu384, **His387**, **Glu411**, Asp415, Tyr523	**His383**, Glu384, **His387**, Phe391, His410, **Glu411**, Asp415, Tyr523	**Glu411**
	AM	-73.216	-	Tyr62, Asn85, Ile88, Ala89, Glu123, Arg124	-

[a] Number in brackets indicates the number of hydrogen bonds formed with the same ACE residue. Residues in the inhibitor binding site of ACE are listed in **bold,** whereas other residues in the active site pockets of ACE are underlined.

The empirical evidence for the anti-DPP-IV and anti-ACE activities of the five bifunctional HFL peptides in this study were previously reported [48–50, 57, 58]. Nevertheless, their modes of ACE inhibition are largely unknown, except for VF, which was reported as a competitive ACE inhibitor [49]. ACE inhibitory peptides that bind to a site on ACE other than its active site are non-competitive inhibitors [65]. Thus based on their interactions with ACE (Table 6**)**, GW possibly also acts as a competitive inhibitor, like VF. By contrast, PH, GY, and AF could be potential non-competitive inhibitors. Based on the comparison of docking scores, GW and PH may bind more stably to ACE when compared with all three reference peptides. Interestingly, the relative docking scores of the three reference peptides also correlate with their relative anti-ACE activities, as reported by Mohd and Gan [13].

Moreover, CF is potentially a non-competitive inhibitor. It was predicted to bind to residues outside the active site pockets, which contrasts with the binding interactions formed by KM, a potential competitive inhibitor. Furthermore, we also found that PH, which could not bind to the active site pockets of ACE, was predicted to form more stable binding to ACE

relative to VF. Similarly, among the reference peptides, CF, which could not bind to the active site pockets of ACE, was predicted to bind more stably to ACE, when compared with KM. KM is the only reference peptide that could bind to the S1 active site pocket and the inhibitor binding site of ACE (Table 6).

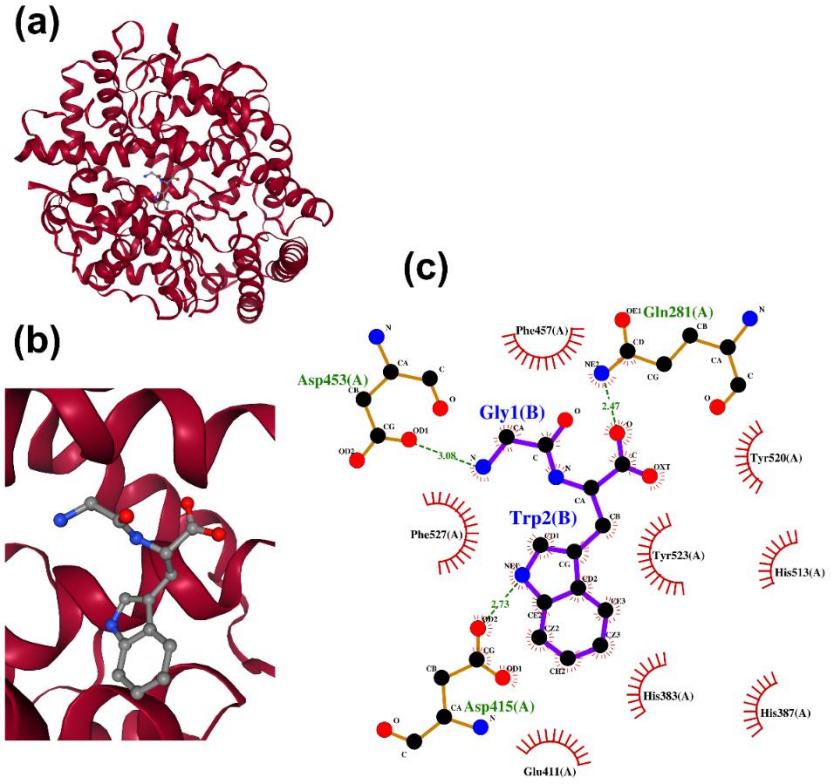

Figure 3. The ACE-GW docked model presented in 3D (**A**, **B**) and 2D (**C**) diagrams. Descriptions for 2D and 3D diagrams are the same as those in **Figure 2**.

Taken together, our docking results suggest that all five bifunctional peptides derived from HFL are likely competitive DPP-IV inhibitors, but consist of a mixture of competitive and non-competitive ACE inhibitors; similar observations were made on the three reference peptides we used for comparison.

This being an *in silico* study has its limitations. For instance, the release of bioactive peptides by *in silico* GI digestion in this study does not consider factors such as *in vivo* GI enzyme concentration, temperature, duration, and accessibility of enzyme to the cleavage sites of proteins [66, 67]. Thus, *in vitro* simulated GI digestion experiments are warranted to confirm the release of the aforementioned bifunctional peptides from HFL proteins. *In vitro* and/or *in vivo* validation of the predicted lack of toxicity and allergenicity, high GI absorption, and plasma half-life of the peptides is also inevitable. Notwithstanding, this study has narrowed down the potential peptide candidates to only five, which should make the task more feasible. Going forward, the possibility of synergism between the five bifunctional peptides is an interesting question to address, which could not be investigated with our *in silico* strategy.

4. Conclusions

Five bifunctional anti-DPP-IV + anti-ACE peptides were discovered from major HFL proteins following *in silico* GI digestion. The five peptides were predicted to be non-toxic and non-allergenic, besides a high probability of GI absorption. Molecular docking simulations point to the five peptides being competitive DPP-IV inhibitors and a mixture of competitive

and non-competitive ACE inhibitors. Taken together, our study suggests that HFL proteins could be a promising source of bifunctional peptides with antidiabetic and antihypertension potential.

Funding

This research received no external funding.

Acknowledgments

None declared.

Conflicts of Interest

The authors declare no conflict of interest.

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