Wiley
Journal of Food Biochemistry
Volume 2026, Article ID 4629120, 13 pages
https://doi.org/10.1155/jfbc/4629120

WILEY

Research Article

Isolation, Identification and Characterization of Two Novel Peptide Inhibitors of Dipeptidyl Peptidase 4 From Mung Bean (*Vigna radiata*) Protein Hydrolysate: Gastric Stability and Mechanistic Insight

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Received 11 April 2025; Revised 14 December 2025; Accepted 17 December 2025

Academic Editor: Akhilesh K. Verma

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The growing global burden of diabetes warrants search for natural, food-derived bioactive molecules, including peptides. In this paper, dipeptidyl peptidase 4 (DPP4), a type 2 diabetes drug target,inhibitory peptides from the seed protein hydrolysates of mung bean (*Vigna radiata*) were studied. The peptides were isolated using activity-guided purification and identified by LC-MS/MS. Two peptides: VSVPHKDAE (MPHPep1) and VAPPGKGEVR (MPHPep2) showed DPP4 inhibition via competitive and uncompetitive modes, respectively. The average K_i value of MPHPep1 and MPHPep2 was found to be 1.40 mm and 0.23 mm, respectively. Both peptides were stable towards gastric digestion conditions. While MPHPep1 is stable towards intestinal digestion, MPHPep2 exhibited 64% loss in its activity. Moreover, the peptides are stable with DPP4 and retain near-complete inhibition activity after incubation with DPP4 for 30 min. In vitro DPP4 inhibition observed in cell-based assays using the Caco-2 cell line further validated the inhibitory potential of these peptides. Finally, the possible interactions of MPHPep1 and MPHPep2 with DPP4 using molecular docking tool are discussed.

Keywords: bioactive peptides; diabetes; DPP4 inhibitors; mung bean; protein hydrolysates

1. Introduction

Diabetes poses a significant global health challenge, impacting nearly 387 million individuals worldwide, and out of this, 90% of cases are of Type 2 diabetes [1]. Developing countries bear the major brunt, with countries like India being dubbed as diabetes epicentres [2]. Among different treatment modalities, dipeptidyl peptidase 4 (DPP4), a serine protease, is recognized as a key therapeutic target for diabetes. Since the enzyme is the primary agent that metabolizes incretin hormones like glucagon-like peptide 1 (GLP1) and gastric inhibitory peptide (GIP), responsible for insulin stimulation, inhibitors of the enzymes like gliptins would prolong the postprandial levels of these hormones, thereby leading to insulin stimulation for a longer duration after meals [3]. Nonetheless, the administration of synthetic inhibitors like gliptins has been associated with several adverse effects [4–11]. Owing to these, exploring DPP4 inhibitory substances from natural sources is an interesting avenue for research.

Bioactive peptides—short amino acid sequences derived from natural sources—possess specific biological activities with potential health benefits. These sequences might be encrypted in naturally occurring protein sequences and could be released either during chemical/enzymatic hydrolysis or by microbial fermentation [12]. Among different bioactivities,

inhibitory activities of the DPP4 enzyme by peptides have been well established [13]. Given this, protein hydrolysates and bioactive peptides obtained from food proteins have gained increased attention and are deemed safe supplements for modulating glycemic responses [13]. Numerous DPP4 inhibitory peptides, demonstrating their potential to aid diabetes management, have been reported from food sources [13–17] as well as food processing by-products [18, 19]. This underscores the untapped potential of diversity of food sources, including by-products, in the generation of DPP4 inhibitory and other bioactive peptides. Although less effective than drug-based DPP4 inhibitors, bioactive peptides may offer numerous advantages over the latter, like fewer side effects and lower costs [14]. Recent years have witnessed a burgeoning interest in peptide-based therapeutic agents [20], and during 2015–2019, US FDA's approval of 15 peptide-based drugs signifies their growing importance, representing 7% of the total drugs approved within that period.

Legumes, which serve as an excellent source of proteins, peptides and phytochemicals, have also been reported to provide DPP4 inhibitory peptides [21–23], which could be released from the legume proteins by commercial proteolytic enzymes like alcalase and flavourzyme, or by human digestive enzymes, *viz*., pepsin, pancreatin, trypsin and chymotrypsin [24]. Mung bean (*Vigna radiata*) is a pulse of considerable global dietary importance, particularly in Asian countries like India, as a source of protein. BIOPEP-UWM database analysis [25] of biologically active peptides suggests it can be a good source of DPP4 inhibitory peptides. Recently, mung bean has been suggested as a potential source for DPP4 inhibitory peptides [26], but a systematic activity-based separation, identification and characterization of such peptides is lacking, based on the available literature.

In the present study, DPP4 inhibition potential of mung bean seed protein hydrolysate prepared using alcalase enzyme was assessed. The inhibitory peptides were subsequently purified using size-based fractionation, and separation of peptides was achieved using C18 column. Two DPP4 inhibitory peptides were identified—VSVPHKDAE (MPHPep1) and VAPPGKGEVR (MPHPep2)—and were characterized in terms of gastrointestinal stability, stability towards digestion by DPP4 and mechanism of inhibition. The inhibitory potential of the peptides was also evaluated using an intestinal cell line–based DPP4 activity.

2. Materials and Methods

2.1. Materials. Porcine kidneys, used in DPP4 purification, were acquired from a nearby slaughterhouse. Alcalase 2.4 L (2.4 AU/g) was purchased from Novo Nordisk (Denmark). Gly-Pro-7-amido-4-methylcoumarin hydrobromide (Gly-Pro-AMC), pepsin, bovine pancreatic trypsin and α-chymotrypsin were obtained from Sigma Chemicals, USA. Enzymatic activities of the gastrointestinal enzymes were confirmed according to the methodologies described in INFOGEST 2019 [27]. Media and bovine serum for cell culture were purchased from HiMedia Laboratories, Mumbai, India.

2.2. Methods

2.2.1. Preparation of Mung Bean Seed Protein Isolate and Hydrolysate. The isolation of mung bean seed protein isolate was achieved through isoelectric precipitation [28]. In brief, aqueous suspension (10% w/v in distilled water) of finely ground mung bean seed was made alkaline (pH 9) using NaOH to extract the proteins. After 2 h of constant stirring at room temperature and maintaining the pH, the insoluble seed material was removed by centrifugation at 5000 g for 10 min. Further, the pH of the supernatant was adjusted to 4 by adding concentrated HCl to precipitate the proteins. The protein pellet was recovered, lyophilized, and stored at −20°C until required. The protein hydrolysate was prepared by hydrolysing aqueous suspension (10% w/v) of protein isolate using alcalase (1% v/v) for 6 h at pH 8.0 at 50°C with constant stirring. The suspension was heat-inactivated post-incubation (90°C for 10 min) and centrifuged (10,000 g for 30 min). The supernatant finally obtained was named as mung bean protein hydrolysate (MPH) and was kept at −20°C until further use.

2.2.2. Purification of DPP4 Enzyme From Pig Kidney. DPP4 was purified using modified published methods [29, 30]. A brief overview and the SDS-PAGE profile of the purified protein are shown in Figure S1 (Supporting information, doc file).

2.2.3. DPP4 Enzyme Assay. The activity of DPP4 was quantified by a fluorescence-based assay, utilizing Gly-Pro-AMC as the substrate, as described in reference [31], with some modifications. The assay system (50 μL) consists of 20-μM Gly-Pro-AMC and DPP4 enzyme (10 ng) in 50-mM Tris buffer (pH 8.0). The reaction mixture was incubated at 37°C for 5 min, and the reaction was stopped using 950 μL of stopping buffer (acetate buffer pH 4.0). For inhibition assay, the peptide at specific concentration was incubated with enzyme followed by the addition of substrate. Enzyme activity was measured with respect to the release of AMC, quantitated by fluorescent measurement (excitation: 360 nm, emission: 460 nm) using a plate reader (BioTek Synergy, USA). The percentage residual DPP4 activity was calculated as follows:

$$\%\ \text{DPP4 residual activity} = \left[\frac{\text{DPP4 acitivty in the presence of inhibitory fraction}}{\text{activity of uninhibited DPP4}}\right] \times 100. \quad (1)$$

2.2.4. Isolation and Identification of DPP4-Inhibitory Peptides

2.2.4.1. Fractionation of MPH. MPH, after thawing, was fractionated via ultrafiltration in stirred cells, employing a 1-kDa membrane. The fractions obtained were designated as 1-kDa retentate and 1-kDa permeate and were evaluated for DPP4 inhibition.

2.2.4.2. Reversed-Phase Chromatography. The ultrafiltration fraction (1 kDa permeate) exhibiting maximum DPP4 inhibitory activity was further subjected to RP-HPLC using a Quaternary pump (PU-2089 Plus, Jasco, Japan) and Hypersil ODS C18 Reverse Phase column (4.6 × 250 mm, 5 μM, Thermo Scientific, USA). Mobile phase A comprised 0.1% trifluoroacetic acid (TFA) in deionized water, and Mobile phase B comprised 0.1% TFA in acetonitrile. The samples were centrifuged before being injected into the column to remove suspended particles. Elution was performed using a 30-min linear gradient of 0%–60% Mobile phase B at a flow rate of 1 mL/min. Absorbance at 220 nm was monitored using a UV detector (Knauer, Germany). For further rounds of purification, a narrower gradient (0%–40%) was utilized for better resolution. At each step, the fractions were collected, freeze-dried, reconstituted with deionized water and DPP4 inhibition potential was assessed.

2.2.4.3. Peptide Identification and Synthesis. The RP-HPLC fraction exhibiting maximal DPP4 inhibitory activity was subjected to identification by LC-MS/MS employing a Thermo Scientific nanoLC-Orbitrap system (MS Facility, C-CAMP, Bangalore, India). The samples were desalted, lyophilized and reconstituted prior to injection into the LC system for separation. MS and MS/MS data were acquired, and peptide identification was performed using specialized proteomics software (ProteinPilot).

The peptides corresponding to the identified sequences were commercially synthesized by standard solid phase synthesis (GL Biochem, Shanghai, China), and their purity (> 95%) was confirmed with mass spectrometry.

2.2.5. Evaluation of Gastrointestinal Stability of Peptides and Resistance Towards Cleavage by DPP4. To determine the gastrointestinal stability of the identified DPP4 inhibitory peptides, an in vitro digestion protocol was employed, wherein MPHPep1 and MPHPep2 were sequentially treated with pepsin, trypsin and chymotrypsin to simulate the enzymatic processes of the gastrointestinal tract. Initially, the peptides were incubated for 1.5 h at 37°C in a buffer (pH 1.6) containing pepsin at a concentration of 1% (w/w). Thereafter, the pH was neutralized by the addition of 1 M Tris buffer pH 8.0 to a final concentration of 50 mM, followed by the addition of trypsin and chymotrypsin at a concentration of 1% (w/w) and further incubated for 1.5 h at 37°C. Finally, the enzymes were heat-inactivated at 90°C for 10 min. In order to rule out the possibility of loss of inhibition potential of the peptides during incubation and heating, control reactions containing only buffer without any of the enzymes were subjected to similar treatment. The resulting reaction mixtures were then used for assessing DPP4 inhibition using the procedure described previously, such that the final concentration of the peptides in the assay was 500 μM.

To determine whether the peptides were stable towards digestion by DPP4, MPHPep1 (1 μL) and MPHPep2 (2 μL) (both from 10 mM stock) were individually incubated with 25 μL of DPP4 buffer at 37°C for 30 min. Control reaction containing only DPP4 was also used to account for possible loss, if any, of DPP4 activity upon incubation. This was followed by assessing DPP4 activity by the addition of substrate.

2.2.6. Determination of DPP4 Inhibition Mechanism and K_i for the Peptides. Michaelis–Menten kinetics studies were performed to deduce the various kinetic parameters—K_m, V_{max} and K_i (for competitive inhibition: enzyme (E)–inhibitor (I) complex dissociation constant; for uncompetitive inhibition: enzyme (E)–substrate (S)–inhibitor (I) complex dissociation constant). To this end, DPP4 activity was assessed as described earlier using increasing concentrations of Gly-Pro-AMC (5–400 μM). The resulting data was analyzed using nonlinear regression model for Michaelis–Menten kinetics in GraphPad Prism 9.0 (GraphPad Software, Inc.). An assessment of the K_m and V_{max} in the absence and presence of MPHPep1 and MPHPep2 suggested the DPP4 inhibition mode of the peptides. By deducing the apparent K_m (K_m^{app}) and V_{max} (V_{max}^{app}) in the presence of inhibitory peptides, the K_i values were determined from the following relationships:

For competitive inhibition:

$$K_m^{app} = \alpha K_m; \tag{2}$$

For uncompetitive inhibition:

$$K_m^{app} = \frac{K_m}{\alpha}, \qquad V_{max}^{app} = \frac{V_{max}}{\alpha}; \tag{3}$$

where $\alpha = 1 + [I]/K_i$ and $[I]$ is the inhibitor (peptide) concentration.

2.2.7. Assessment of DPP4 Inhibition of the Peptides Using Human Intestinal Cell Line. Caco-2 cells (obtained from NCCS, Pune, India) (passage numbers 42–50) were seeded (2×10^4) in multiwell plates with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and incubated for 24 h. Subsequently, media supernatant was taken out and the cells were washed once with Hanks' balanced salt solution (HBSS). The inhibitory potential of MPHPep1 and MPHPep2 towards cell surface DPP4 enzyme was assessed at two different concentrations, viz. 100 and 500 μM, using Gly-Pro-AMC substrate at concentrations of 10 and 200 μM. The reaction was then allowed in a CO_2 incubator at 37°C for 30 min, followed by fluorescence measurement as described previously.

2.2.8. Interaction of Peptides With DPP4 Analyzed Using Molecular Docking Tool. GalaxyPepDock (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=PEPDOCK) [32] was utilized to dock MPHPep1 against DPP4 structure (PDB: 1NU6, Chain A). GalaxyPepDock predicts three-dimensional structures of protein–peptide complexes from input protein structure and peptide sequence, employing a combination of database-derived interaction information and energy optimization. For MPHPep2, the docking was executed using HPEPDOCK 2.0 server (https://huanglab.phys.hust.edu.cn/hpepdock/) [33], with DPP4 structure (PDB: 1NU6, Chain A) as the target. In both the cases, input peptide sequences were submitted as FASTA files. The highest scoring models for MPHPep1-DPP4 and MPHPep2-DPP4 complexes were further analyzed using the molecular visualization tools PyMol [34] and UCSF Chimera [35]. 2D protein–ligand interaction plots were generated by LigPlot [36].

2.2.9. Statistical Analysis. The values presented as mean ± SEM are derived from three replicates. Statistical analyses and enzyme kinetics parameters estimation were performed using GraphPad Prism 9.0 (GraphPad Software, Inc.)

3. Results and Discussion

3.1. Purification of the Peptides and Their Identification. Mung bean seeds are rich in protein, and therefore, it can be a potential source for bioactive peptides [37]. Some of the reported ones include those showing antihypertensive effect via ACE enzyme inhibition [38]. A preliminary analysis of the mung bean seed proteins on the BIOPEP-UWM database (https://biochemia.uwm.edu.pl/en/biopep-uwm-2/) [25] suggests that it might contain potent DPP4 inhibitory peptides (Supporting file 1, Excel sheet). To release such potential DPP4 inhibitory peptides from the mung bean protein isolate, it was enzymatically hydrolysed using Alcalase, which is a commercially available protease widely used to release biologically active peptides [39].

The hydrolysate exhibited strong DPP4 inhibitory activity, when monitored at different time intervals during hydrolysis (Figure S2, Supporting information, Word document). The hydrolysate prepared after 6 h of protease digestion showed saturation of inhibitory effect (~90% DPP4 activity inhibition) (Figure S2, Supporting information, Word document); hence, it was used for further purification. As the enzyme-generated hydrolysate might yield peptides of diverse lengths, and because DPP4 inhibitory peptides are generally reported to be between 2 and 15 amino acids in length [13], size-based fractionation (employing a 1-kDa ultrafiltration membrane) was performed. The results showed that the 1-kDa permeate had a similar DPP4 inhibitory activity to that of the unfractionated hydrolysate (input), suggesting that peptides ≤ 1 kDa (~≤ 10 amino acids) accounted for the majority of the DPP4 inhibitory activity of the MPH (Figure 1(a)).

The 1-kDa fraction was subjected to further resolution by RP-HPLC using C18 column (Figure 1(b)). DPP4 activity assay performed using the fractions from RP-HPLC suggested that the one corresponding to 15–16 min retention time showed the highest DPP4 inhibition (Figure 1(c)). The fraction was designated as MPH 4.6 and was chosen for further purification using a narrower mobile phase gradient (0%–40% acetonitrile), to enable a better resolution and separation of peptides. A total of 12 regions were collected (4.6.1 to 4.6.12; Figure 1(d)) and each was assessed for its ability to inhibit DPP4 activity. Fraction 4.6.6 exhibited the highest DPP4 inhibitory activity (Figure 1(e)) and also eluted as a single peak in RP-HPLC (Figure 1(f)). Analysis of the peak by LC-MS/MS identified three peptides: VSVPHKDAE, FVDAQPQQKQ and VAPPGKGEVR (Table 1). The identified peptides were custom-synthesized. Amongst those, VSVPHKDAE and VAPPGKGEVR showed potent DPP4 inhibition, which is consistent with the fact that most DPP4 inhibitory peptides reported are Val, Ala or Pro rich (Supporting file 2, Excel sheet), and were thus chosen for further characterization and designated as MPHPep1 and MPHPep2, respectively.

3.2. Gastrointestinal Stability of the Peptides and Their Stability Towards Cleavage by DPP4. The susceptibility of bioactive peptides and hydrolysates to degradation within the gastrointestinal tract is a key factor limiting their efficacy. Analyses using Expasy PeptideCutter server (https://web.expasy.org/peptide_cutter/) were performed to predict the susceptibility of the peptides to gastrointestinal enzymes, viz., pepsin, trypsin and chymotrypsin. Both MPHPep1 and MPHPep2 did not have a pepsin cleavage site, indicating gastric stability. MPHPep1 had one cleavage site each for trypsin and chymotrypsin (Figure 2(a), inset), whereas MPHPep2 had one cleavage site for trypsin (Figure 2(b), inset).

To further determine the stability of the peptides in the gastrointestinal environment, they were subjected to simulated gastric digestion (SGD) using pepsin and simulated gastrointestinal digestion (SGID) using pepsin and trypsin + chymotrypsin in sequential manner. For MPHPep1, the results suggested a marginal reduction (< 10%) in the inhibitory potential upon subjecting to pepsin digestion (SGD) conditions, i.e., with and without pepsin reactions both showed decrease compared to the control reaction, with the difference between no treatment and SGD being nonsignificant (Figure 2(a)). Similarly, trypsin and chymotrypsin (SGID) treatment also showed a slight decline (< 10%) in the inhibitory potential of MPHPep1. It is worth noting that MPHPep1 could retain > 90% of its inhibitory potential upon subjecting to GI digestion, while MPHPep2 exhibited a decrease in its inhibitory activity by 64%, mainly after digestion with trypsin and chymotrypsin enzymes (SGID) (Figure 2(b)), suggesting that its digestion products possessed lower/no residual DPP4 inhibition. This is in agreement with HPLC profiles of the two peptides obtained post enzyme treatments—wherein MPHPep1 did not show a substantial change in the profile upon pepsin (SGD) treatment, and only a marginal peak reduction following trypsin + chymotrypsin treatments (SGID) (Figure 2(c)), MPHPep2 displayed two new peaks with lower retention

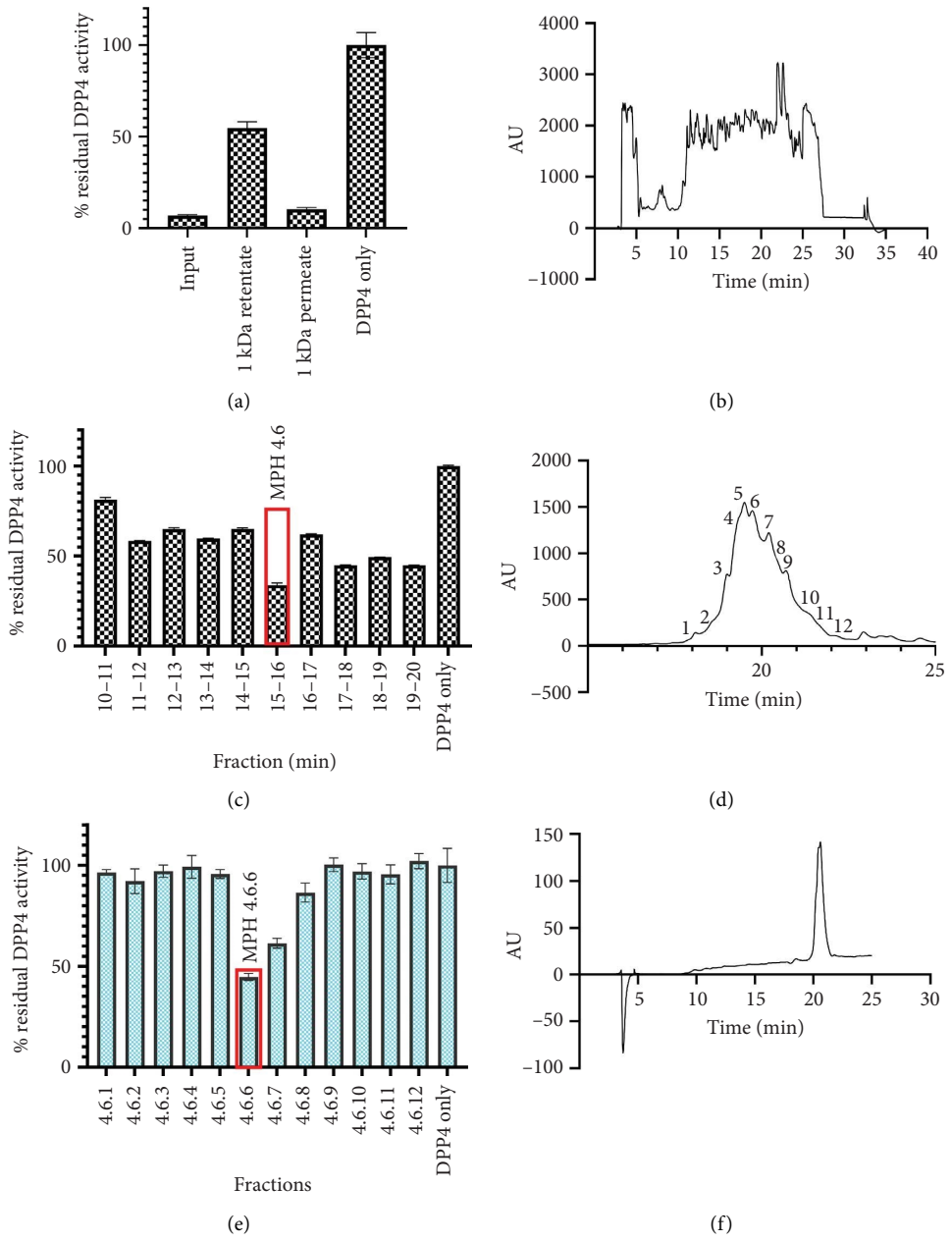


Figure 1: Purification of DPP4 inhibitory peptides from mung bean seed protein hydrolysates: (a) DPP4 inhibitory activity of different fractions obtained after size-based fractionation of MPH. The values are expressed as the mean ± SEM of three independent experiments. (b) RP-HPLC profile of 1-kDa permeate of MPH. (c) DPP4 inhibitory activity of different fractions obtained after RPHPLC of 1-kDa permeate of MPH. The maximum activity exhibited by fraction eluted at 15–16 min is labelled as MPH 4.6. (d) RP-HPLC profile of MPH 4.6. (e) DPP4 inhibitory activity of different fractions of MPH 4.6; the maximum activity exhibited by fraction 4.6.6 is labelled as MPH 4.6.6. (f) RP-HPLC profile of MPH 4.6.6.

Table 1: Identification of peptides in RPHPLC purified fraction of MPH using LC-MSMS.

Sequence	Obs MW	Obs m/z	Theor MW	Theor m/z	Best conf (peptide)
VSVPHKDAE	980.493103	491.2538	980.49274	491.2536316	99
FVDAQPQQKQ	1187.591064	594.8028	1187.5935	594.8040161	99
VAPPGKGEVR	1008.572449	505.2935	1008.5717	505.2930908	98.4

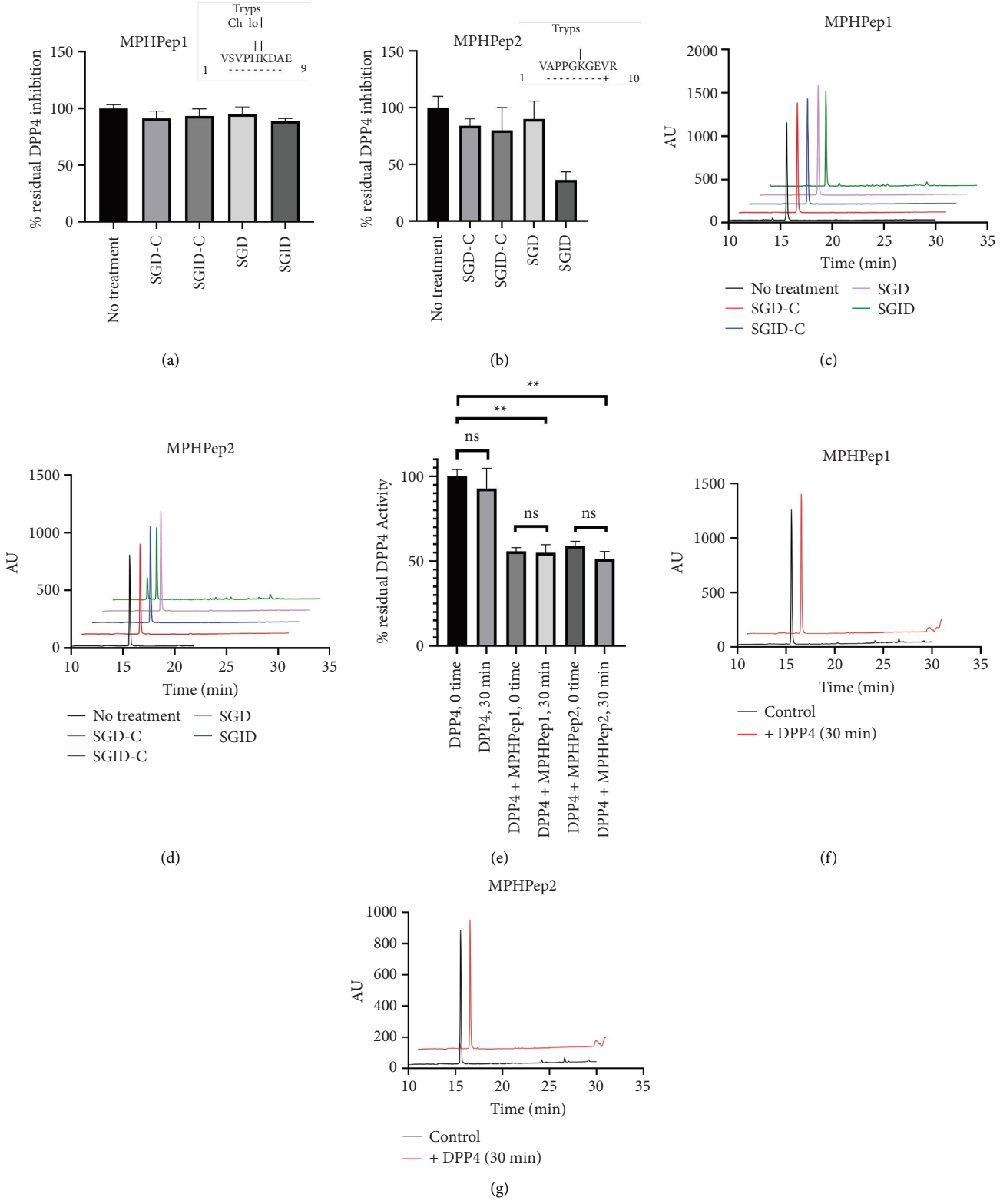


Figure 2: Gastrointestinal stability of the peptides and their stability towards cleavage by DPP4: Residual DPP4 activity of MPHPep1 (a) and MPHPep2 (b) subjected to SGD and SGID conditions. Cleavage sites for the peptides by gastrointestinal enzymes pepsin, trypsin, and chymotrypsin, as analyzed using Expasy peptide cutter, are depicted in the inset. The respective chromatograms of MPHPep1 (c) and MPHPep2 (d) are shown. Stability towards cleavage by DPP4 in terms of residual DPP4 activity (e) and HPLC profiles of MPHPep1 (f) and MPHPep2 (g) following incubation with DPP4 are depicted. The values are expressed as the mean ± SEM of three independent experiments. **: $p \leq 0.01$.

times and reduced peak heights (Figure 2(d); SGID). Preservation of inhibitory potential, as ascertained from the control reactions (SGD-C and SGID-C) in which the peptides alone were subjected to pH 1.6 and heat, suggests the stability of the peptides against acidic conditions and heat. These results, especially pertaining to MPHPep1, are akin to a recently published study that reported peptides from tilapia viscera hydrolysate, which could retain DPP4 inhibitory activity after SGID conditions [40].

Many of the reported DPP4 inhibitory peptides have Val, Ala and Pro at the N-terminus and thus may be important for the inhibition of the enzyme [16, 17]. However, it is interesting to note that MPHPep2, which has a trypsin cleavage site at the sixth position, shows a substantial reduction in inhibition potential upon trypsin + chymotrypsin (SGID) treatment, suggesting that residues after the cleavage site (-GEVR) might be crucial for the inhibitory activity of the peptides.

Prodrugs and substrate-type peptide inhibitors are cleaved by their target enzymes, releasing a more or less potent inhibitor than the original molecule [16]. On the other hand, true inhibitors are not acted upon by the enzymes, and such peptides retain their inhibitory activity even after having been incubated with the enzyme. To assess whether MPHPep1 and MPHPep2 are stable towards the cleavage by DPP4, they were incubated with DPP4 for 30 min at 37°C. Both the peptides preserved their inhibitory activity post incubation with DPP4 (Figure 2(e)). This was corroborated by the retention of the HPLC peak profiles after incubation with DPP4 (Figures 2(f) and 2(g)). Stability with the target enzyme is a vital aspect that is neglected in many studies and should be given due consideration while assessing the bioactivity of any given peptide. Competitive-type peptide inhibitors might also act as a substrate to the enzyme. For instance, DPP4 inhibitory peptides like diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) are susceptible to DPP4 degradation [41]. However, diprotin A and diprotin B retain strong inhibitory potential even after incubation with DPP4. Nevertheless, it is also possible that some peptides may lose their inhibitory ability after being degraded [13, 42]. For instance, slight loss of inhibitory activities of two peptides from tilapia (LPCL and TPFLPDE) after preincubation with recombinant human DPP4 also suggests that such peptides can be of substrate type [40]. Initially, it was believed that DPP4 cleaves only after Pro or Ala at the P1 site, but the extended substrate spectrum includes dehydroproline, glycine, hydroxyproline, leucine, serine, threonine or valine [43], suggesting that inhibitory peptides with these residues can act as a substrate for the enzyme. Given these possibilities, it is prudent to assess the stability of a bioactive peptide with DPP4. Our results suggest that the peptides MPHPep1 and MPHPep2 are not degraded by DPP4, or they might be "slow substrates" for this enzyme [44].

3.3. Determination of DPP4 Inhibition Mechanism and K_i for the Peptides. Enzyme inhibitors, including peptides, can be of competitive, noncompetitive and uncompetitive in nature

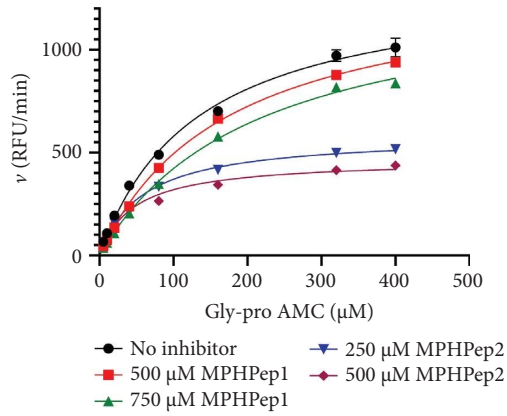


Figure 3: Kinetic studies of DPP4 in the presence of peptides: Michaelis–Menten nonlinear regression of the DPP4 activities in the absence and presence of 500 μM & 750 μM MPHPep1, and 250 μM & 500 μM MPHPep2; performed with various concentrations of substrate. The values are expressed as the mean ± SEM of three independent experiments.

[45]. To elucidate the mode of DPP4 inhibition by MPHPep1 and MPHPep2, DPP4 activity was measured across a range of substrate concentrations, and the data were analyzed using Michaelis—Menten nonlinear regression model (Figure 3). MPHPep1 exhibited a competitive-type inhibition evident by the similar V_{max} and increased K_m value compared to the control (no peptide), with the average K_i for the enzyme–inhibition (EI) complex to be 1397 μM. On the other hand, a decrease in both the K_m and V_{max} with MPHPep2 suggests a uncompetitive mode of inhibition for this peptide, with the average K_i value for the enzyme–substrate–inhibitor (ESI) complex to be 230 μM (Table 2).

3.4. Assessment of DPP4 Inhibition of the Peptides Using the Intestinal Cell Line Caco-2. Owing to the high expression of DPP4 on the surface of intestinal epithelial cells [46], it is pragmatic to assess DPP4 inhibitory peptides using intestinal cell lines [47, 48]. Intestinal cells express brush border proteases on their surface, and these enzymes might play an important role in the modification of DPP4 inhibitory peptides and their transportation to the basolateral side of Caco-2 cells monolayer [49]. Given these, an assessment of the in situ DPP4 inhibition by MPHPep1 and MPHPep2 would corroborate their functionality. To this end, the DPP4 inhibition by MPHPep1 and MPHPep2 was assessed on adherent Caco-2 cells. Two different substrate (Gly-Pro-AMC) concentrations (10 μM and 200 μM) were used, given the different modes of inhibition of the peptides. At both substrate concentrations, MPHPep1 showed higher DPP4 inhibitory activity than MPHPep2 (Figure 4). Inhibition potential was more pronounced at a lower substrate concentration, which is concordant with the competitive mode of inhibition as ascertained from the in vitro kinetics analyses.

To ascertain whether both the peptides are differently degraded or internalized by cells, HPLC analysis of the peptides post incubation with adherent Caco-2 cells was

Table 2: Kinetic parameters of DPP4 in the presence of peptide inhibitors.

	K_m (μM) ± SEM	V_{max} (RFU min^{-1}) ± SEM	K_i (μM)
No inhibitor	131.97 ± 12.13	1343.21 ± 48.49	—
500 μM MPHPep1	174.10 ± 7.70	1355.21 ± 25.96	1566.28
750 μM MPHPep1	212.61 ± 19.02	1320.26 ± 55.02	1227.26
250 μM MPHPep2	57.52 ± 3.39	583.18 ± 10.45	193.13
500 μM MPHPep2	45.97 ± 6.95	466.38 ± 20.21	267.26

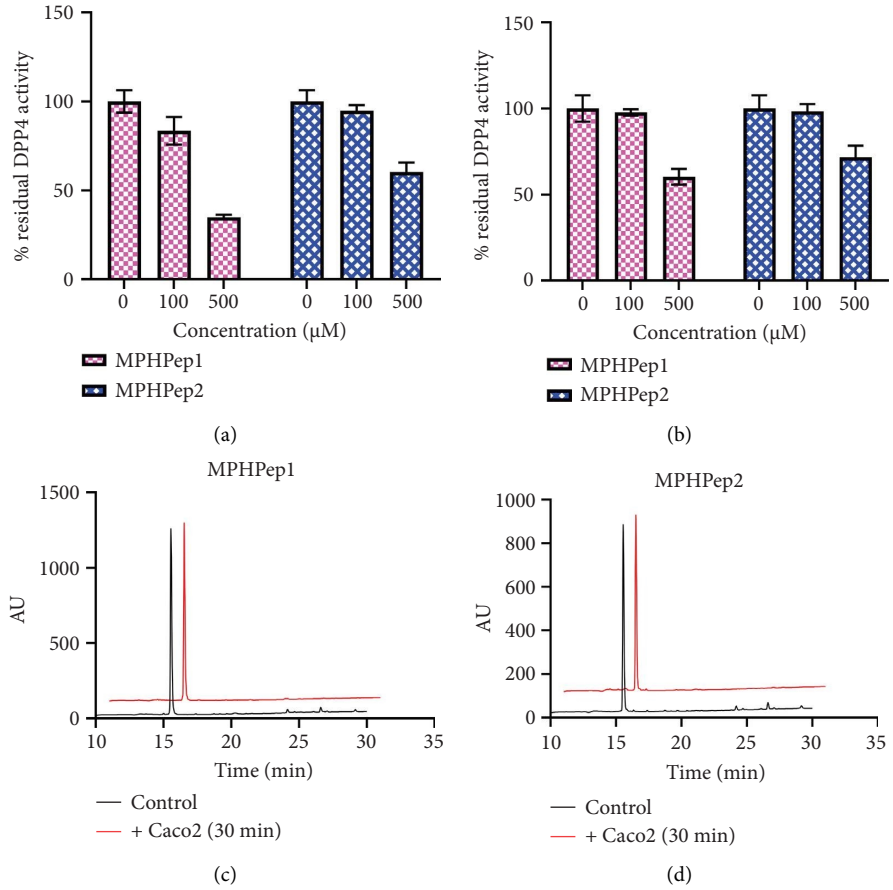


Figure 4: DPP4 inhibitory activity of MPHPep1 and MPHPep2 using adherent cell line Caco-2: The assays were performed at (a) 10 μM and (b) 200 μM concentrations of the substrate using two different peptide concentrations—100 and 500 μM. HPLC chromatograms showing the peptide profiles for (c) MPHPep1 and (d) MPHPep2 after incubation with adherent Caco-2 cells. The values are expressed as the mean ± SEM of three independent experiments.

done. Despite the known expression of various membrane-bound proteases in Caco-2 cells, no significant changes in the peptide profiles were observed. This suggests that lower DPP4 inhibition efficiency of MPHPep2 at both substrate concentrations may be due to the interaction of this peptide with some other molecular target on the surface of the Caco-2 cells. However, the results confirm that the peptides are resistant to degradation by cell surface proteases of Caco-2 cells.

3.5. Interaction of Peptides With DPP4 Analyzed Using Molecular Docking. Molecular docking is a helpful tool for predicting ligand–receptor interactions. Since MPHPep1 showed a competitive inhibition, local docking against DPP4 structure was sought. Tools used for small ligand–protein docking (e.g., Autodock) may not be very reliable for peptides beyond a few amino acids in sequence and necessitate manually placing peptide conformations in the active site [50, 51]. On the other hand, template-guided docking procedures utilize the known structures of protein–peptide complexes to conjecture the unknown peptide–protein complex structure. GalaxyPepDock (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=PEPDOCK) is the most popular template-based docking method and was used to predict MPHPep1-DPP4 interactions [32]. In a comparative analyses of various protein–peptide docking methods (template-based, local and global), GalaxyPepDock

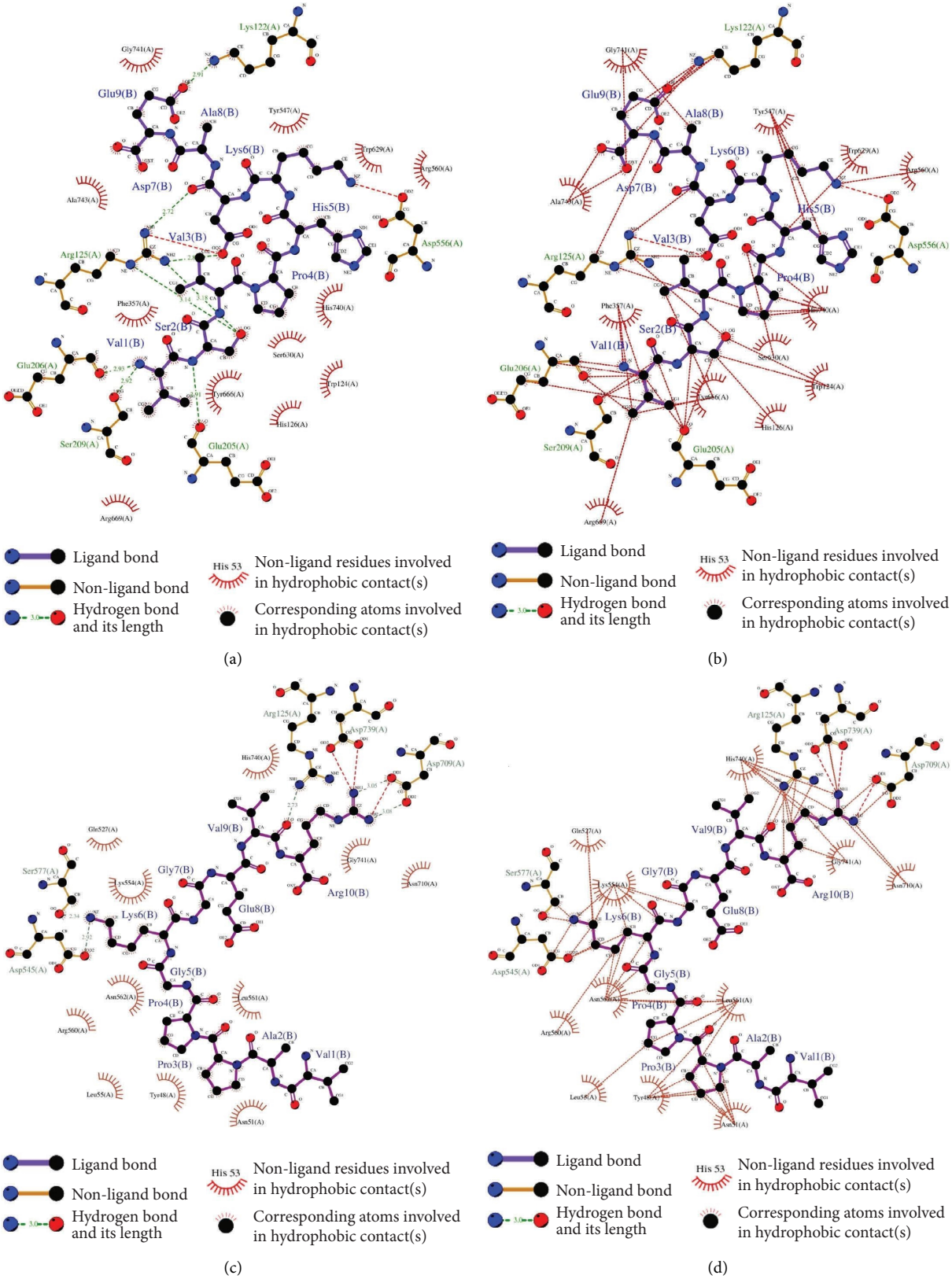


Figure 5: Interaction of peptides with DPP4 analyzed using molecular docking: Interaction plot obtained by LigPlot of MPHPep1-DPP4 interactions–bonded interactions (a) and nonbonded interactions (b). Interaction plot obtained by LigPlot of MPHPep2-DPP4 interactions–bonded interactions (c) and nonbonded interactions (d).

performed the best when a benchmark dataset of peptides, 5–20 amino acids in length, was used for assessment, compared to other popular methods like HADDOCK, CABS-dock, etc. [52]. In addition, the availability of experimental structures of DPP4 bound with drug and peptide ligands rationalized the choice of template-based docking for our analyses. The GalaxyPepDock method is reliable beyond the receptor TM score of 0.7 [53].

MPHPep1 was seen to occupy the active site of DPP4 through the interfacial opening between the β-propeller and α/β-hydrolase domains (Figure S3(A), Supporting information, Word document). Analyses of the docking results using LigPlot suggested the possible interactions between the inhibitory peptides and DPP4 (Figure 5). Lys6 of MPHPep1 can interact electrostatically with Asp556 in a loop connecting a β-strand and a helix in the α/β-hydrolase domain. Glu9 of MPHPep1 might interact with Lys122 of the β-propeller domain via H-bonding interaction. Ser2 and Asp7 of MPHPep1 can form H-bonds with Arg125 of DPP4, while the side chain of Asp7 can interact electrostatically as well. The main chain amide group of Ser2 of MPHPep1 can interact electrostatically with Glu205 of DPP4, while Val1 of MPHPep1 can form hydrogen bonds with Glu206 and Ser209 of DPP4 via the N-terminal amine group (Figure 5(a)). Arg125, Glu204, Glu205 and Glu206 are part of the S2 pocket of DPP4 which are responsible for recognizing and binding with the N-terminal residue of the substrate and are indispensable for its enzymatic activity [54–57]. Nonbonded interactions are detailed in Figure 5(b). In particular, MPHPep1 can form hydrophobic interactions with , Tyr666, Tyr547 and Phe357 of the S1, S1' and S2 pockets, respectively, as well as Ser630 and His740 of the catalytic triad of DPP4. These possible interactions suggested by the docking results indicate a strong MPHPep1–DPP4 interaction.

Since MPHPep2 showed an uncompetitive mode of inhibition, it might act outside the substrate binding site of DPP4. HPEPDOCK server was utilized to assess plausible MPHPep2-DPP4 interactions for two reasons: (i) it performed better than other global protein–peptide docking algorithms when tested against a benchmark dataset [52]; (ii) it accounts for peptide flexibility and evaluates multiple peptide conformations against the receptor structure, along with scoring the top models.

The docking analyses suggested that MPHPep2 might interact with the residues lining the cavity at the β-propeller and α/β-hydrolase domain interface, with peptide ends protruding away from the active site (Figure S3(B)). Even though MPHPep2 could interact via nonbonded interactions with Asn710 and His740 of the catalytic triad, extensive interactions with the S2 pocket residues, like those seen with MPHPep1, were not observed (Figures 5(c) and 5(d)). Arg10 of MPHPep2 can form ionic interactions with Asp709 and Asp739, and H-bonding with Asp709, of DPP4, similar to what was observed for sitagliptin and metformin in a previous report [58]. Asp709 and Asp739 are present in two loops connecting two different α-helices and β-strands in the α/β hydrolase domain of DPP4. Val9 of MPHPep2 can make hydrogen-bonding contact with Arg125 of DPP4 in the loop connecting two β-strands in the β-propeller domain. Lys6 of MPHPep2 can make an H-bond interaction with Asp545 in a β-strand in the α/β hydrolase domain of DPP4.

4. Conclusion

Management of Type 2 diabetes by dietary intervention is a promising research avenue, and bioactive peptides have been proposed to be effective mediators for the same. Mung bean seed protein hydrolysate exhibited potent DPP4 inhibitory activity. Two peptides, VSVPHKDAE (MPHPep1) and VAPPGKGEVR (MPHPep2), were isolated by activity-guided fractionation and displayed robust DPP4 inhibition. MPHPep1 showed excellent gastrointestinal stability, while MPHPep2 exhibited a loss in inhibitory potential after trypsin–chymotrypsin treatment. Both peptides were stable against DPP4 cleavage. Kinetic analysis revealed MPHPep1 as a competitive inhibitor and MPHPep2 as an uncompetitive inhibitor. Both peptides demonstrated efficacy in inhibiting cell surface DPP4 in Caco-2 cells. This study highlights the potential of these peptides for diabetes management, warranting further in vivo and cellular investigations.

5. Limitations of the Study

The major limitation of our study is that the bioavailability of DPP4 inhibitory peptides reported here has not been assessed. Further studies in murine model would help to evaluate the efficacy/potential of the peptides or MPH to manage impaired glucose tolerance of the animal. Although cell surface DPP4 expression in the intestine is more relevant for incretin degradation, soluble DPP4 does exist and inhibition of serum DPP4 would require peptides to be transported across the gut epithelium. Assessment of the incretin hormones' modulation by the peptides would also corroborate their importance at the physiological level, as suggested in a previous study using the murine enteroendocrine cell line STC-1 [19].

Nomenclature

DPP4	Dipeptidyl peptidase 4
Gly-Pro-AMC	Gly-Pro-7-amido-4-methylcoumarin hydrobromide
MPH	Mung bean protein hydrolysate
RP-HPLC	Reversed-phase high-performance liquid chromatography
SGD	Simulated gastric digestion
SGID	Simulated gastrointestinal digestion

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Gaurav Srivastava contributed to methodology, investigation, formal analysis and writing–original draft. Dharmendra K. Maurya contributed to methodology. Sahayog N. Jamdar contributed to conceptualization, methodology and writing–review and editing.

Funding

No funding was received for this manuscript.

Supporting Information

Additional supporting information can be found online in the Supporting Information section. *(Supporting Information)*

Supporting files: The following files are provided as supporting information:

Supporting File 1: DPP4 inhibitory peptides from Major Seed Proteins of Mung BioPep (Excel sheet).

Supporting File 2: DPP4 inhibitory peptides from BioPep (Excel sheet).

Supporting Information: Additional experimental details and results are provided (DOC). Figure S1(A). Overview of the purification protocol for DPP4 from Porcine kidney. (B) SDS-PAGE profile of the purified DPP4.

Figure S2. DPP4 inhibition activity during the hydrolysis of mung protein isolate. Figure S3. Surface presentation of binding of the peptides to DPP4 (PDB: 1NU6, Chain A): (A). For MPHPep1 (B). For MPHPep2.

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