

Article

Stable Non-Competitive DPP-IV Inhibitory Hexapeptide from *Parkia timoriana* Seeds: A Candidate for Functional Food Development in Type 2 Diabetes

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Abstract

The tree bean (*Parkia timoriana*), an underutilized legume valued for its nutritional profile, represents a potential source of bioactive peptides for diabetes management. To our knowledge, this is the first study to identify and characterize DPP-IV inhibitory peptides derived from tree bean seed protein hydrolysates. The tree bean proteins were digested with trypsin, thermolysin, chymotrypsin, pepsin, and simulated gastrointestinal (SGI) enzymes, among which SGI hydrolysis yielded the highest degree of hydrolysis (14%) and strongest DPP-IV inhibitory activity ($IC_{50} = 1289 \pm 58 \mu\text{g/mL}$). Guided by DPP-IV inhibitory assays, sequential fractionation using strong cation exchange and RP-HPLC yielded the most potent fraction, H5, with an IC_{50} of $949 \pm 50 \mu\text{g/mL}$. After peptide identification and synthesis, APLGPF (AF6) emerged as the most potent inhibitor, with an IC_{50} of $396 \pm 18 \mu\text{M}$. Enzyme kinetics revealed a non-competitive inhibition mechanism, corroborated by molecular docking, which indicated binding at an allosteric site of DPP-IV. Furthermore, AF6 remained stable under simulated gastrointestinal digestion and enzymatic exposure, highlighting its resistance to proteolysis. Taken together, these findings highlight *P. timoriana* as an underexplored source of peptides with DPP-IV inhibitory activity and identify AF6 as a promising lead for developing functional foods or nutraceuticals aimed at type 2 diabetes management.

Keywords: *Parkia timoriana*; active peptide; peptide stability; diabetes; natural resources; functional food



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1. Introduction

The global demand for nutraceutical foods continues to rise as individuals increasingly seek natural and functional dietary approaches to support health and prevent chronic diseases [1]. Legumes, in particular, have emerged as a valuable class of nutraceuticals due to their rich composition of bioactive compounds, including proteins, fibers, vitamins, minerals, and antioxidants [2]. Their wide availability, affordability, and nutritional benefits

make legumes a staple in diverse diets around the world. Notably, legumes can provide up to 50% dietary protein, positioning them as an essential component of sustainable and cost-effective nutrition. The health-promoting properties of legumes are deeply rooted in traditional knowledge and have been passed down through generations across various cultures [3].

Indonesia, with its remarkable biodiversity and multi-ethnic heritage, is home to a wide range of legume-based foods that have long been utilized for both nutritional and medicinal purposes. Among these, *Parkia timoriana* (tree bean, locally referred to as kedawung in Indonesia) is widely consumed by the Javanese—the largest ethnic group in the country—as both a functional snack and a traditional remedy [4]. In addition to its nutritional and bioactive potential, *P. timoriana* has long been cultivated in traditional home gardens across Northeast India, where families maintain trees for household consumption and sale of surplus pods, thereby supporting both biodiversity conservation and community livelihoods [5]. Future development of *P. timoriana*-derived functional foods should be aligned with such sustainable practices to ensure ecological sustainability while providing socio-economic benefits. Studies have reported that tree bean seeds possess various biological activities, including antioxidants, antibacterial, anticancer, antidiabetic, antiproliferative, and insecticidal effects [2,6]. Given the growing global prevalence of diabetes—projected to affect 700 million people by 2045 [7]—there is increasing interest in discovering sustainable nutraceuticals for its prevention and management. *Parkia timoriana* (tree bean) exhibits diverse bioactivities, yet studies on its antidiabetic peptides remain scarce. To address this gap, we explored its seed proteins as a source of novel peptides with DPP-IV inhibitory activity, aiming to uncover their potential role in diabetes management.

Diabetes mellitus is a chronic metabolic disorder characterized by impaired insulin production or insulin resistance, resulting in elevated blood glucose levels [8]. While several studies have investigated the antidiabetic potential of bioactive compounds from different *Parkia* species, most have focused on the inhibition of α -glucosidase and α -amylase—enzymes responsible for carbohydrate digestion—by phytochemicals such as phenolics and other small molecules [9–11]. However, there has been limited exploration of DPP-IV inhibitory peptides derived specifically from *P. timoriana*, a plant of both cultural and therapeutic relevance to the Javanese community. DPP-IV rapidly inactivates the incretin hormones GLP-1 (glucagon-like peptide-1) and GIP (glucose-dependent insulintropic polypeptide). Inhibition of DPP-IV prolongs the activity of these hormones, thereby enhancing insulin secretion, suppressing glucagon release, and ultimately improving glycemic control [12,13].

This study aimed to explore, screen, and identify peptides with potential DPP-IV inhibitory activity from *P. timoriana* seed protein. The protein will be isolated and subjected to enzymatic hydrolysis using different proteases, followed by sequential bioassay-guided fractionation based on DPP-IV inhibitory activity. Peptides from the most active fractions will be identified through liquid chromatography–tandem mass spectrometry (LC-MS/MS). Selected peptides will be synthesized to verify their activity and further examined using enzyme kinetic assays and molecular docking to assess their inhibitory mechanism and interaction with DPP-IV. This study may contribute to the ongoing search for natural bioactive peptides as candidates for nutraceutical development targeting type 2 diabetes. In addition, the utilization of *P. timoriana*, a legume with cultural and nutritional significance in Indonesia, may offer insights into the potential of underexplored plant sources for future functional food or pharmaceutical applications. However, information on the bioavailability and in vivo efficacy of peptides from *P. timoriana* remains limited, underscoring the need for further research to assess their physiological relevance.

2. Materials and Methods

2.1. Materials and Chemicals

Raw tree bean (*Parkia timoriana*) seeds, the primary material used in this study, were obtained from a local farmer in Malang City, East Java, Indonesia.

The following chemicals and reagents were used throughout the study: acrylamide, ammonium persulfate (APS), Tris-HCl, and tetramethylethylenediamine (TEMED), required for SDS-PAGE, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trypsin (from bovine pancreas), α -chymotrypsin (from bovine pancreas), pepsin (from porcine gastric mucosa), and thermolysin were sourced from Sigma Chemical Co. Sodium bicarbonate (NaHCO_3), hydrochloric acid (HCl), and sodium chloride (NaCl) were obtained from J.T. Baker (Phillipsburg, NJ, USA). Sodium dodecyl sulfate (SDS), sodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were also purchased from Sigma Chemical Co. Acetonitrile (ACN) was acquired from J.T. Baker (Deventer, The Netherlands), and trifluoroacetic acid (TFA) was purchased from Alfa Aesar (Lancashire, UK).

For bioassays and enzyme studies, human dipeptidyl peptidase-IV (DPP-IV), glycine-proline-p-nitroaniline (GP-pNA), p-nitroaniline (p-NA), and linagliptin were obtained from Sigma Chemical Co. Deionized water (ddH_2O) was prepared using the PURELAB[®] water purification system (ELGA LabWater, Lane End, High Wycombe, UK).

2.2. SDS-PAGE Profiling and Protein Extraction of Tree Bean Seeds

Tree bean seeds were ground into powder, and proteins were extracted using 1% SDS at a 5:1 (*v/w*) ratio. Sonication (40's on/20's off, 30% amplitude, 5 min) enhanced cell disruption before centrifugation (4000 rpm, 15 min). Proteins were precipitated with 10% TCA-acetone (4 °C, 12 h), washed with chilled acetone, and lyophilized. Protein yield was expressed relative to seed powder weight. SDS-PAGE (12.5% resolving gel, 4% stacking gel) was used to assess protein composition, with molecular weights calibrated against a 10–170 kDa marker.

2.3. Enzymatic Hydrolysis of Tree Bean Seed Proteins

Protein isolates were digested with pepsin, trypsin, α -chymotrypsin, thermolysin, or a simulated gastrointestinal (SGI) system. All reactions used a 1:50 (*w/w*) enzyme-to-substrate ratio, except for the SGI intestinal phase (1:100, *w/w*). Conditions were: trypsin/ α -chymotrypsin (pH 8.5, 50 mM NaHCO_3 , 37 °C, 16 h); thermolysin (same buffer, 60 °C, 16 h); pepsin (pH 1.2, 50 mM NaCl, 37 °C, 16 h). SGI digestion included pepsin pre-treatment (2 h, 37 °C, pH 1.2), neutralization to pH 8.5, followed by trypsin and α -chymotrypsin (4 h, 37 °C). Reactions were stopped by boiling (10 min) and centrifuged (10,000 rpm, 15 min). Hydrolysates were filtered through a 3 kDa MWCO membrane to enrich small peptides. C18 SPE columns were employed to remove residual salts from the hydrolysis buffer according to the manufacturer's instructions. The hydrolysates were then lyophilized for further bioactivity testing. These proteases and hydrolysis conditions were chosen to represent complementary cleavage specificities and have been widely used in previous studies for the generation of bioactive peptides, particularly DPP-IV inhibitory peptides [14,15].

2.4. Determination of Degree of Hydrolysis (DH)

DH was quantified by the TNBS assay in 96-well plates. L-leucine standards were used for calibration. After the enzymatic reaction was stopped, the unfractionated hydrolysates (5 mg/mL), without any filtration step, were mixed with phosphate buffer (pH 8.2) and TNBS, incubated at 50 °C for 1 h in the dark with shaking at 70 rpm, and then quenched

with HCl. Absorbance at 340 nm was recorded. DH (%) was calculated from released peptides relative to total bonds, estimated from protein concentration divided by mean amino acid mass (110 g/mol), as described previously [16].

2.5. DPP-IV Inhibitory Activity Assay

Inhibition was assessed using GP-pNA as substrate. Reactions contained 25 μ L GP-pNA, 25 μ L sample, and 50 μ L DPP-IV (0.5 U/ μ L) in Tris-HCl (pH 8.0), incubated at 37 °C for 1 h, and terminated with acetate buffer (pH 4.0). Linagliptin served as positive control. Absorbance was measured at 405 nm. IC₅₀ values were determined from dose–response curves (GraphPad Prism version 5; GraphPad Software, Solana Beach, CA, USA), as described previously [16].

2.6. Sequential Bioassay-Guided Fractionation TBSP SGI Hydrolysate

The TBSP SGI hydrolysate was subjected to sequential bioassay-guided fractionation using strong cation exchange (SCX) chromatography followed by reverse-phase high-performance liquid chromatography (RP-HPLC).

For the initial SCX step, offline fractionation was performed using two mobile phases: mobile phase A (5% acetonitrile [ACN] + 0.2% formic acid [FA] in deionized water) and mobile phase B (5% ACN + 0.2% FA + 0.5 M NaCl in deionized water). SP Sepharose® beads (Sigma-Aldrich, St. Louis, MO, USA) were loaded into a Pierce™ micro-spin column (Thermo Fisher Scientific, Rockford, IL, USA) and operated in conjunction with an injection pump (LSP01-1A, LongerPump Inc., Baoding, China). The lyophilized TBSP SGI hydrolysate was dissolved in solvent A at a concentration of 10 mg/mL. Fractionation was carried out at a constant flow rate of 20 μ L/min, yielding ten fractions based on increasing percentages of solvent B (FT: 0%B, F1: 5%, F2: 10%, F3: 15%, F4: 20%, F5: 30%, F6: 40%, F7: 60%, F8: 80%, and F9: 100%). Each fraction was desalted, lyophilized, and stored at −20 °C for subsequent DPP-IV inhibitory assays and IC₅₀ determination. The fraction showing the highest DPP-IV inhibition and the lowest IC₅₀ was selected for further purification.

The most active SCX fraction was reconstituted in 5% ACN + 0.1% FA at 50 mg/mL and injected (100 μ L) into an RP-HPLC semi-preparative system equipped with a C18 column (10 mm $\times$ 250 mm, 5 μ m; Thermo Scientific, Waltham, MA, USA). The RP-HPLC gradient was applied as follows (solvent B): 0–5 min, 5–10%; 5–22 min, 10–17%; 22–45 min, 17–30%; 45–50 min, 30–50%; 50–52 min, 50–80%; 52–57 min, 80%; 57–58 min, 80–100%; 58–65 min, 100%. Solvent A consisted of 5% ACN + 0.1% TFA, and solvent B was 95% ACN + 0.1% TFA. The separation was performed at a flow rate of 4 mL/min with UV detection at 214 nm. Collected fractions (H1–H15) were freeze-dried and assessed for DPP-IV inhibitory activity and IC₅₀ values to identify the most potent peptide fraction.

2.7. LC-MS/MS Peptide Analysis, Peptide Synthesis, and In Silico Assessment

The sample was reconstituted in 5% acetonitrile (ACN) containing 0.1% formic acid (FA) in deionized water for LC-MS/MS analysis. A 3 μ L aliquot of a 1 μ g/ μ L sample was injected into a hybrid LTQ Orbitrap XL mass spectrometer operated in heated electrospray ionization (HESI) and positive-ion mode. The MS was operated under the following conditions: sheath gas 40 (arb. units), spray voltage 4 kV, capillary voltage 20 V, capillary temperature 300 °C. Full MS scans were acquired over m/z 150–2000 at a flow rate of 200 μ L/min.

Automated peptide sequencing was performed in PEAKS X+ software version 10.6 (Bioinformatics Solutions Inc., Waterloo, ON, Canada). Parameters: enzyme, simulated gastrointestinal digestion; instrument, LTQ Orbitrap XL; precursor (parent) mass tolerance, 20 ppm; fragment mass tolerance, 0.6 Da; average local confidence (ALC) $\geq$ 50%. All sequences were manually verified. The toxicity of the identified peptides was evaluated

using the ToxinPred web server (<http://crdd.osdd.net/raghava/toxinpred/>, accessed on 19 September 2025). The AlgPred web server (<https://webs.iitd.edu.in/raghava/algpred/>, accessed on 18 September 2025) was used to evaluate the allergenicity of the identified peptides.

Selected peptides were synthesized by microwave-assisted solid-phase peptide synthesis (SPPS) on Wang resin using Fmoc-based chemistry. Coupling, capping, and deprotection steps were performed under microwave irradiation (75 °C, 20 W, 3–5 min); activation employed OxymaPure, DIC, DIPEA, and HBTU. After final deprotection, peptides were cleaved with 95% TFA/3% H₂O/3% TIS at 38 °C for 30 min, precipitated with ice-cold diethyl ether, and lyophilized for further analysis.

2.8. DPP-IV Inhibition Pattern of AF6

To clarify the inhibitory mechanism of AF6 toward DPP-IV, a Lineweaver–Burk plot was constructed by plotting the reciprocals of substrate concentrations (GP-pNA) against the reciprocals of reaction velocities (pNA formation rates). Assays were performed under three conditions: in the absence of inhibitor and in the presence of AF6 at concentrations below and above its IC₅₀ value. The plots were subsequently analyzed to identify the type of inhibition exerted by the peptide.

2.9. Molecular Docking Simulation

Molecular docking simulations were performed to evaluate the binding interactions between human dipeptidyl peptidase-IV (DPP-IV) and the AF6 from tree bean seed protein hydrolysate. Peptide structures were generated using BIOVIA Discovery Studio Visualizer version 2019 (Accelrys Software, Cambridge, UK), and energy minimization was carried out with the CHARMM (Chemistry at HARvard Macromolecular Mechanics) force field applied to both ligands and the receptor. The X-ray crystal structure of human DPP-IV (PDB ID: 1WCY) was obtained from the Protein Data Bank (<https://www.rcsb.org/>; accessed on 10 August 2025). Before docking, native ligands and water molecules were removed.

2.10. Peptide Stability Toward SGI and DPP-IV

The stability of peptides under simulated gastrointestinal (SGI) conditions was evaluated following our previously established protocols [15,16]. The enzyme-to-peptide ratio for all SGI treatments was maintained at 2% (*w/w*). The synthetic peptide APLGPF (AF6) was dissolved in 35 mM NaCl buffer (pH 2.0) and subjected to pepsin digestion at 37 °C for 120 min to simulate gastric protease digestion. The pH was then adjusted to 7–8, and the solution was incubated with trypsin and α -chymotrypsin at 37 °C for 4 h to simulate the intestinal phase, after which the enzymes were inactivated by boiling. The extent of peptide degradation and resistance to SGI treatment was determined using LC–MS analysis.

The resistance of AF6 to DPP-IV was assessed through a pre-incubation assay. Briefly, 25 μ L of AF6 was combined with 50 μ L of DPP-IV solution (0.5 U/ μ L) and incubated at 37 °C for 3 h. Following this pre-incubation, 25 μ L of 1.6 mM GP-pNA, a chromogenic substrate analogue of DPP-IV, was added, and the reaction was allowed to proceed for an additional hour. All reactions were carried out in 0.1 M Tris-HCl buffer (pH 8.0). The enzymatic activity was terminated by adding 100 μ L of 1 M sodium acetate buffer (pH 4.0) to each reaction mixture.

2.11. Statistical Analysis

All measurements were performed in triplicate, and results are expressed as the mean $\pm$ standard error. Data visualization and graph preparation were carried out using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Statistical analyses were

performed in Minitab 18.0 using one-way ANOVA, followed by Tukey's post hoc test for pairwise comparisons. Differences were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. SDS-PAGE Protein Profiling of Tree Bean Seed Protein Isolate

Protein extraction and precipitation from tree bean seed (TBS) powder yielded a dry tree bean seed protein (TBSP) mass equivalent to 30% of the total dry weight (29.9 g TBSP per 100 g TBS powder), consistent with previous reports [3]. The TBSP was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to examine its protein profile. The electrophoretogram (Figure 1) showed multiple distinct bands below 100 kDa, with the most prominent bands appearing between 53 kDa and 70 kDa. The intensity of these bands indicates that proteins within this molecular weight range are the major constituents of the extract. These findings are consistent with earlier studies reporting albumins and globulins (10–70 kDa) as the predominant storage proteins in *P. timoriana* [3,17] and with the high protein content previously described for tree bean seeds [18].

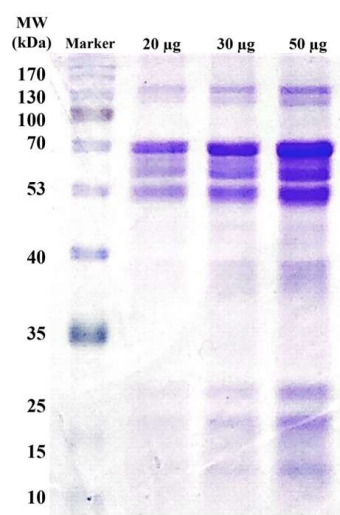


Figure 1. SDS-PAGE analysis of tree bean seed (TBS) protein isolate. Lane 1: protein marker; Lanes 2–4: tree bean seed protein (TBSP) at 20, 30, and 50 µg, respectively.

3.2. Degree of Hydrolysis and DPP-IV Inhibitory Potential of Tree Bean Seed Protein Hydrolysates

Tree bean seed proteins (TBSP) were hydrolyzed using four single enzymes (pepsin, trypsin, α -chymotrypsin, thermolysin) and a simulated gastrointestinal (SGI) system combining pepsin, trypsin, and α -chymotrypsin in sequence. Hydrolysates (TBSPH) were desalted and enriched for <3 kDa peptides to target low-molecular-weight fractions with higher bioactivity potential [19].

The SGI treatment produced the highest degree of hydrolysis (DH, 14%), exceeding all single-enzyme digestions (Figure 2A). This may be attributed to its multi-protease, two-step approach, which more effectively breaks down proteins and releases diverse peptide sequences [20]. As shown in Figure 2B, the DPP-IV inhibitory activity of TBSP hydrolysates was evaluated at a concentration of 2.5 mg/mL. The enhanced activity observed for the SGI hydrolysate may be related to both its high DH [21] and the structural characteristics of the peptides generated.

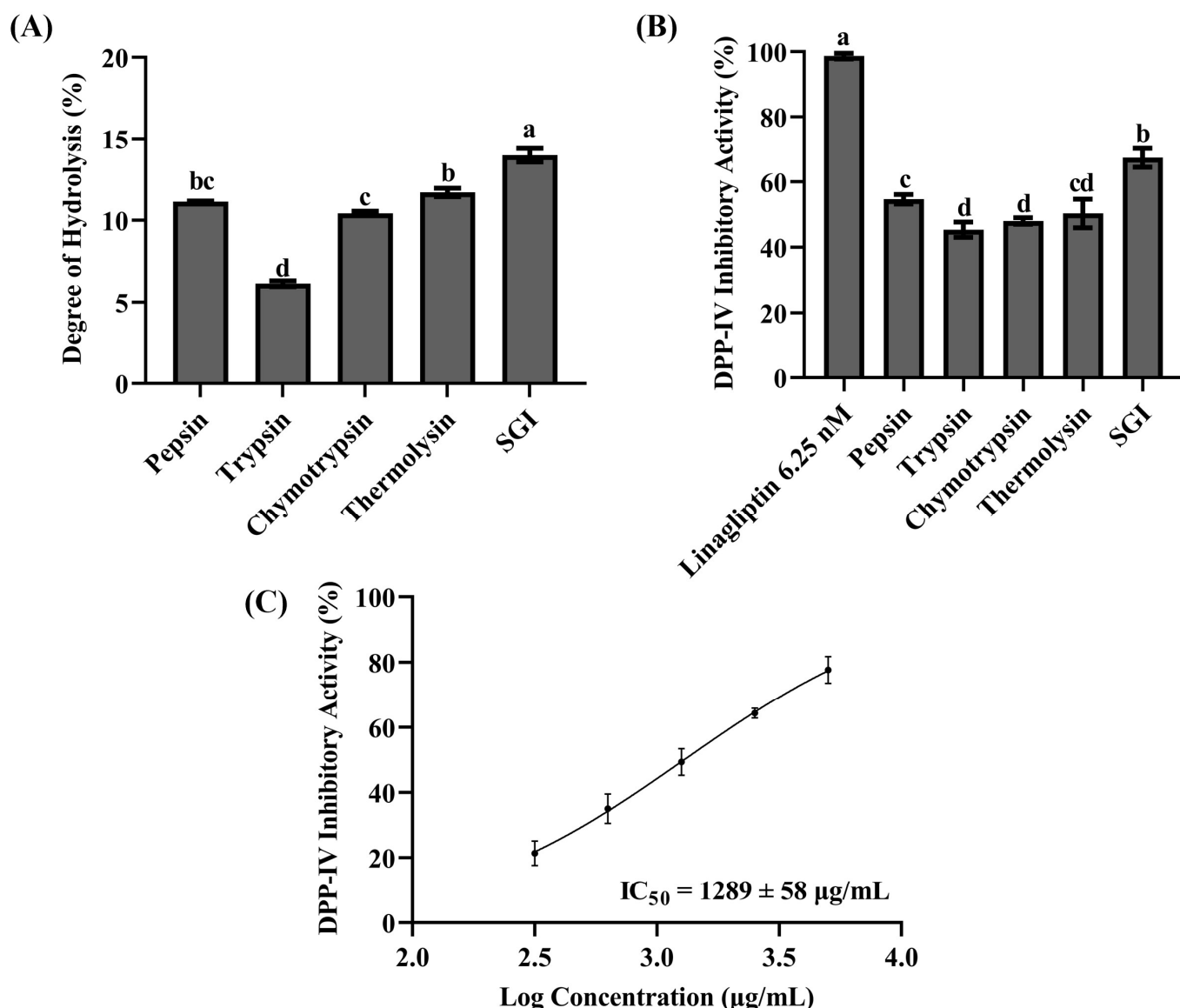


Figure 2. (A) Degree of hydrolysis (DH) and (B) DPP-IV inhibitory activity of tree bean seed protein hydrolysates (TBSPH). Values are expressed as mean $\pm$ SEM ($n = 3$). Bars with different letters indicate significant differences ($p < 0.05$) within each group. (C) Half-maximal DPP-IV inhibitory concentration (IC₅₀) of the TBSP SGI hydrolysate.

The TBSP SGI hydrolysate exhibited a half-maximal DPP-IV inhibitory concentration (IC₅₀) of 1289 ± 58 µg/mL (Figure 2C), indicating a strong inhibitory potential within the range reported for other legume-derived hydrolysates. This IC₅₀ value was lower (indicating greater potency) than those reported for soy (2390 ± 190 µg/mL) and chickpea (2680 ± 180 µg/mL) hydrolysates prepared under comparable enzymatic conditions [22]. It is also slightly more potent than the soybean protein hydrolysate obtained by sequential digestion with porcine pepsin and pancreatin, which exhibited an IC₅₀ of 1490 ± 140 µg/mL [23].

The SGI protease combination (pepsin, trypsin, and α -chymotrypsin) preferentially generates peptides enriched in hydrophobic and basic residues, a characteristic profile of DPP-IV inhibitory peptides [24]. These structural features likely contribute to the observed functional activity. These results underscore the importance of enzymatic strategy in tailoring peptide composition and optimizing bioactivity, supporting TBSP SGI hydrolysate as a promising alternative protein source for producing bioactive peptides with antidiabetic po-

tential. However, future studies on TBSP hydrolysates exploring additional diabetes-related pathways, such as α -glucosidase and α -amylase inhibition, would be of great interest.

3.3. Sequential DPP-IV Assay Guided-Fractionation of TBSP SGI Hydrolysate

The TBSP SGI hydrolysate was fractionated using a two-step separation strategy comprising strong cation exchange (SCX) chromatography followed by reverse-phase high-performance liquid chromatography (RP-HPLC). In the first dimension, SCX fractionation was performed on an SP Sephadex column using solvent A (5% ACN, 0.2% FA) and solvent B (5% ACN, 0.2% FA, 0.5 M NaCl). The hydrolysate, dissolved in solvent A, was eluted stepwise to generate eleven fractions at increasing solvent B concentrations (0–100%). All fractions were desalted via SPE prior to activity testing.

DPP-IV inhibitory activity was evaluated at 2.5 mg/mL with 6.25 nM linagliptin as a positive control. Fraction F1 exhibited the highest inhibitory activity (Figure 3A) and was further analyzed for IC_{50} values, yielding $1121 \pm 66 \mu\text{g/mL}$ (Figure 3B), which was lower than that of the unfractionated SGI hydrolysate, indicating enrichment of active peptides.

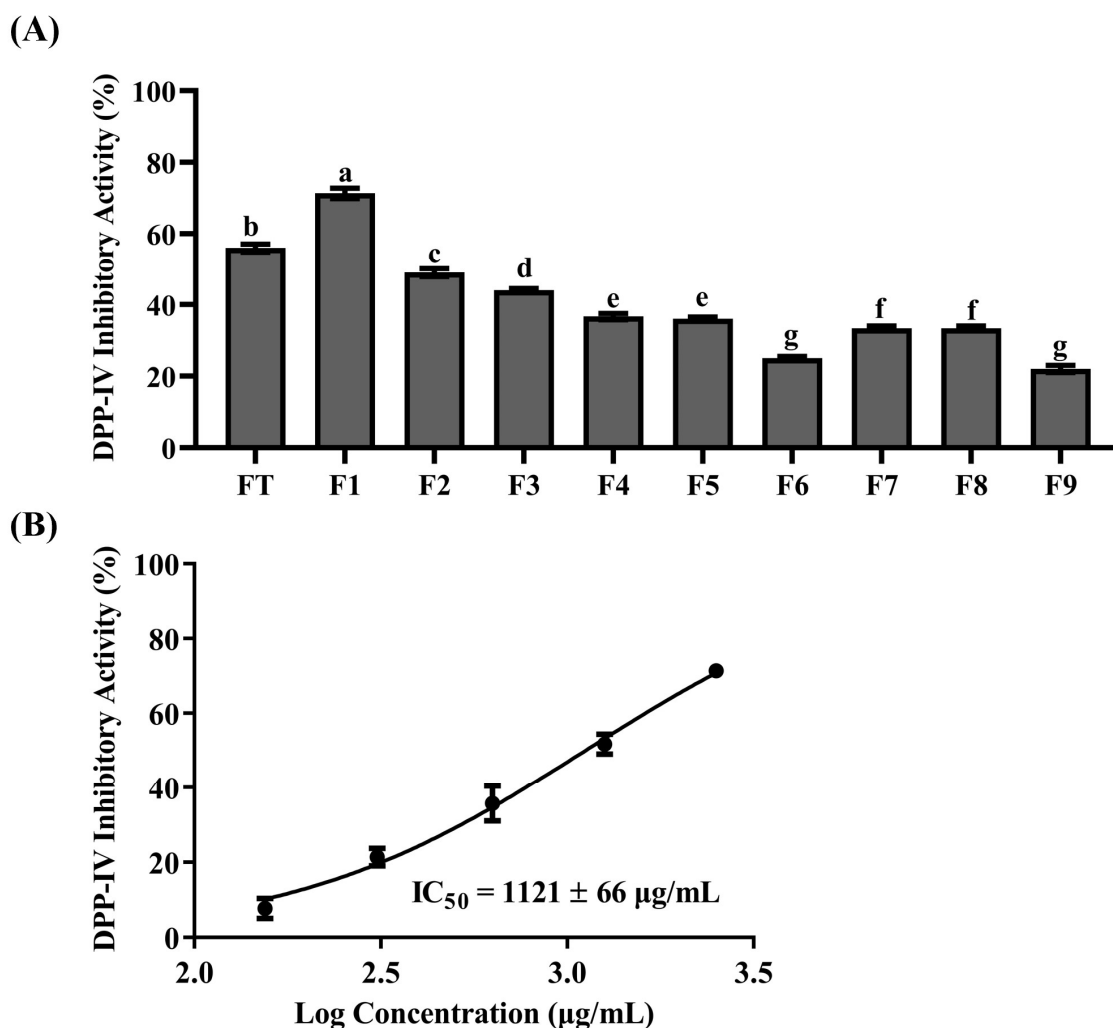


Figure 3. Cont.

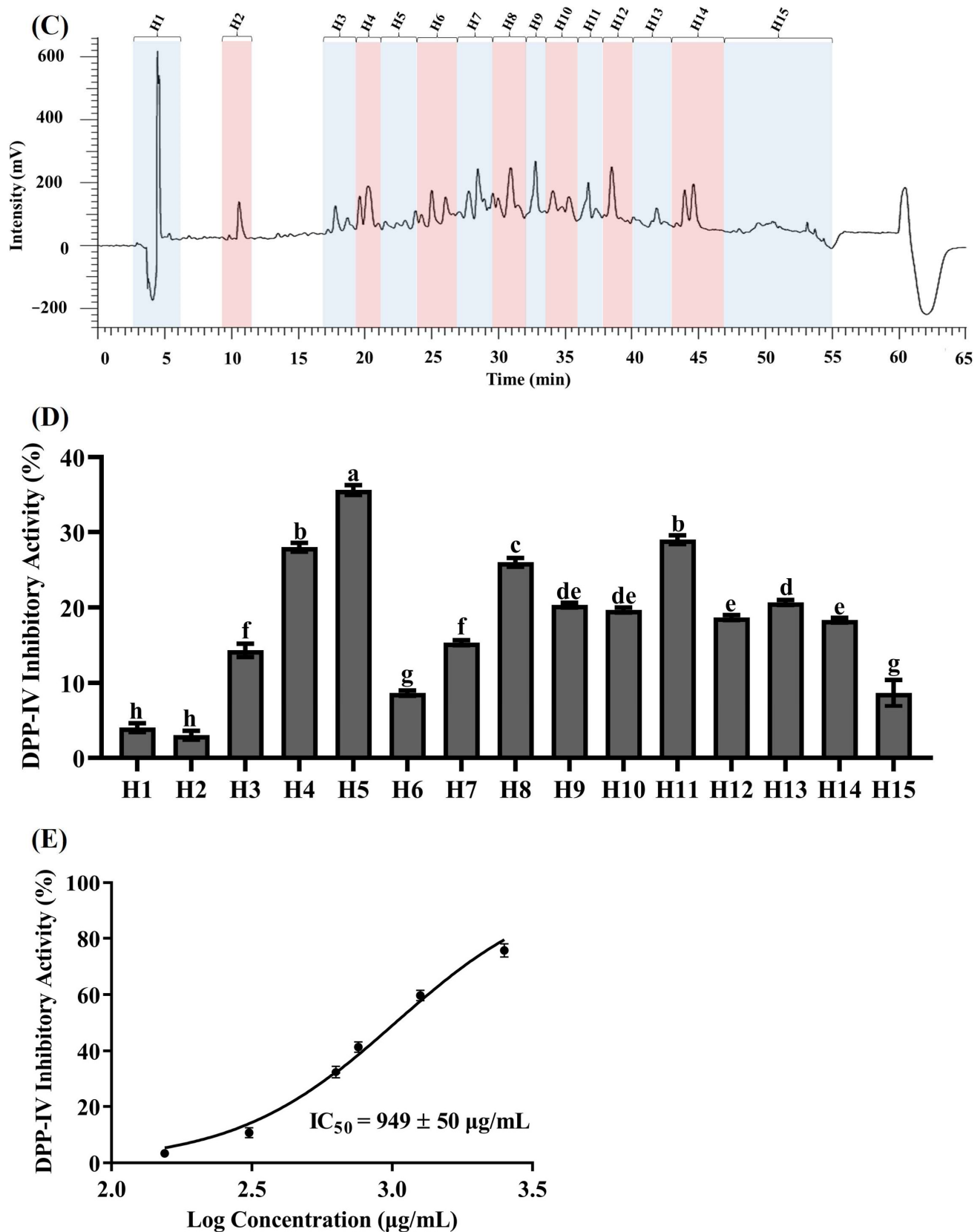


Figure 3. (A) DPP-IV inhibitory activity of SCX fractions, (B) IC_{50} value of SCX fraction F1, (C) RP-HPLC chromatogram of SCX fraction F1, (D) DPP-IV inhibitory activity of RP-HPLC fractions, and (E) IC_{50} value of RP-HPLC fraction H5. Values are presented as mean $\pm$ SEM ($n = 3$). Bars with different letters indicate significant differences within each group ($p < 0.05$).

Since F1 exhibited the strongest activity, it was further fractionated by RP-HPLC on a C18 column. A total of fifteen fractions (H1–H15) were collected (Figure 3C). As shown in Figure 3D, their DPP-IV inhibitory activity was assessed at 0.625 mg/mL, with H5 displaying the highest inhibition (38%). These active fractions were eluted within the 17–30% solvent B (95% ACN, 0.1% TFA) range, indicating that the bioactive peptides are relatively hydrophobic. Among them, H5, the most potent fraction, exhibited an IC_{50} of $949 \pm 50 \mu\text{g/mL}$ (Figure 3E), which was lower than that of the precursor SGI hydrolysate and SCX fraction F1, thereby demonstrating the progressive enrichment of DPP-IV inhibitory peptides through sequential fractionation.

SCX separates peptides by net charge via electrostatic interactions, while RP-HPLC resolves them primarily by hydrophobicity. The combined SCX–RP-HPLC strategy remains a widely applied and effective strategy for enriching bioactive peptides [25]. Collectively, these results suggest that sequential DPP-IV assay-guided fractionation is a reliable method for isolating inhibitory peptides from TBSP SGI hydrolysates. However, LC-MS/MS analysis will be required to elucidate their specific sequences.

3.4. LC-MS/MS Peptide Identification from RP-HPLC Fraction H5 and Bioactivity Confirmation

The most active RP-HPLC fraction (H5) obtained from the two-step sequential fractionation was analyzed by LC-MS/MS using an LTQ-Orbitrap XL to identify peptides with potential DPP-IV inhibitory activity. Sequential fractionation progressively reduces peptide mixture complexity, thereby enriching bioactive candidates. As shown in Figure 4A, the chromatographic profiles of the hydrolysate and the H5 fraction revealed a markedly reduced number of peaks, reflecting decreased sample complexity. From the high-intensity peaks in H5, three peptides were identified: APLGPF (m/z 601.3337; top panel), FVLNAPY (m/z 823.4339; middle panel), and DTLGLGVSNL (m/z 988.5298; bottom panel) (Figure 4B). Moreover, in silico assessment indicated that these peptides are non-toxic and non-allergenic.

The DPP-IV inhibitory activities of APLGPF, FVLNAPY, and DTLGLGVSNL were further validated using synthetic peptides. At a concentration of 2.5 mg/mL, all three peptides exhibited measurable inhibitory effects (Figure 4C). Among them, APLGPF (AF6) showed the highest inhibition, with an IC_{50} value of $396 \pm 18 \mu\text{M}$ (Figure 4D). Notably, AF6 demonstrated greater antidiabetic potential than pumpkin seed-derived peptides such as LPGF ($IC_{50} = 451.49 \mu\text{M}$), LPGFF ($IC_{50} = 449.68 \mu\text{M}$), and MPLPA ($IC_{50} = 478.88 \mu\text{M}$) [26], as well as stronger DPP-IV inhibitory activity than several previously reported tetra- and pentapeptides, including DPLV ($IC_{50} = 698 \mu\text{M}$) [27], VLGP ($IC_{50} = 580.4 \mu\text{M}$) [24], and ADHPF ($IC_{50} = 1660 \mu\text{M}$) [28]. Although AF6 is slightly less potent than LPFYFN, TFFPQ, and ATFFPQ ($IC_{50} = 70.2, 176.9, 268.3 \mu\text{M}$, respectively) derived from coix seed [29], it nevertheless exhibited significantly higher DPP-IV inhibitory efficacy compared to KKSSG ($IC_{50} = 1266 \mu\text{M}$), CPGNK ($IC_{50} = 1681 \mu\text{M}$), and GGGLHK ($IC_{50} = 1075 \mu\text{M}$), which were derived from Mexican black and Brazilian Carioca beans [30]. However, future studies on TBSP hydrolysates and AF6 that explore additional diabetes-related pathways, such as α -glucosidase and α -amylase inhibition, would be of considerable interest. Subsequently, a strong AF6 signal was observed in both the hydrolysate and fraction H5, and confirmed by comparison with the synthetic AF6, supporting its role as the primary peptide responsible for the DPP-IV inhibitory activity of the TBSP SGI hydrolysate (Figure 4A).

The Ala¹ and Pro² residues at the N-terminus of AF6 (Ala¹-Pro²-Leu³-Gly⁴-Pro⁵-Phe⁶) may be critical for its inhibitory activity of DPP-IV, owing to the presence of Ala or Pro at the penultimate position [31]. Previous studies have also suggested that peptides with N-terminal hydrophobic amino acids (Ala, Val, Leu, Ile, Met, Phe, Trp) and the presence of Pro or Ala at the second position are favored for DPP-IV inhibition [15,16,31,32]. AF6 (Ala¹-Pro²-Leu³-Gly⁴-Pro⁵-Phe⁶) features an aliphatic N-terminal residue, a proline at the

second position, hydrophobic residues in the central region, and an aromatic phenylalanine at the C-terminus. A Pro² residue flanked by Leu, Val, Phe, Ala, or Gly is considered a favorable structural motif for DPP-IV inhibitory peptides [33]. These structural features are likely to contribute significantly to its DPP-IV inhibitory activity. However, large-scale optimization of hydrolysate or active peptide production, cost production, product formulation, and evaluation of bioactivity should be considered in future studies to support practical applications. The inhibition mechanism of AF6 and its intermolecular interactions with DPP-IV will be examined further.

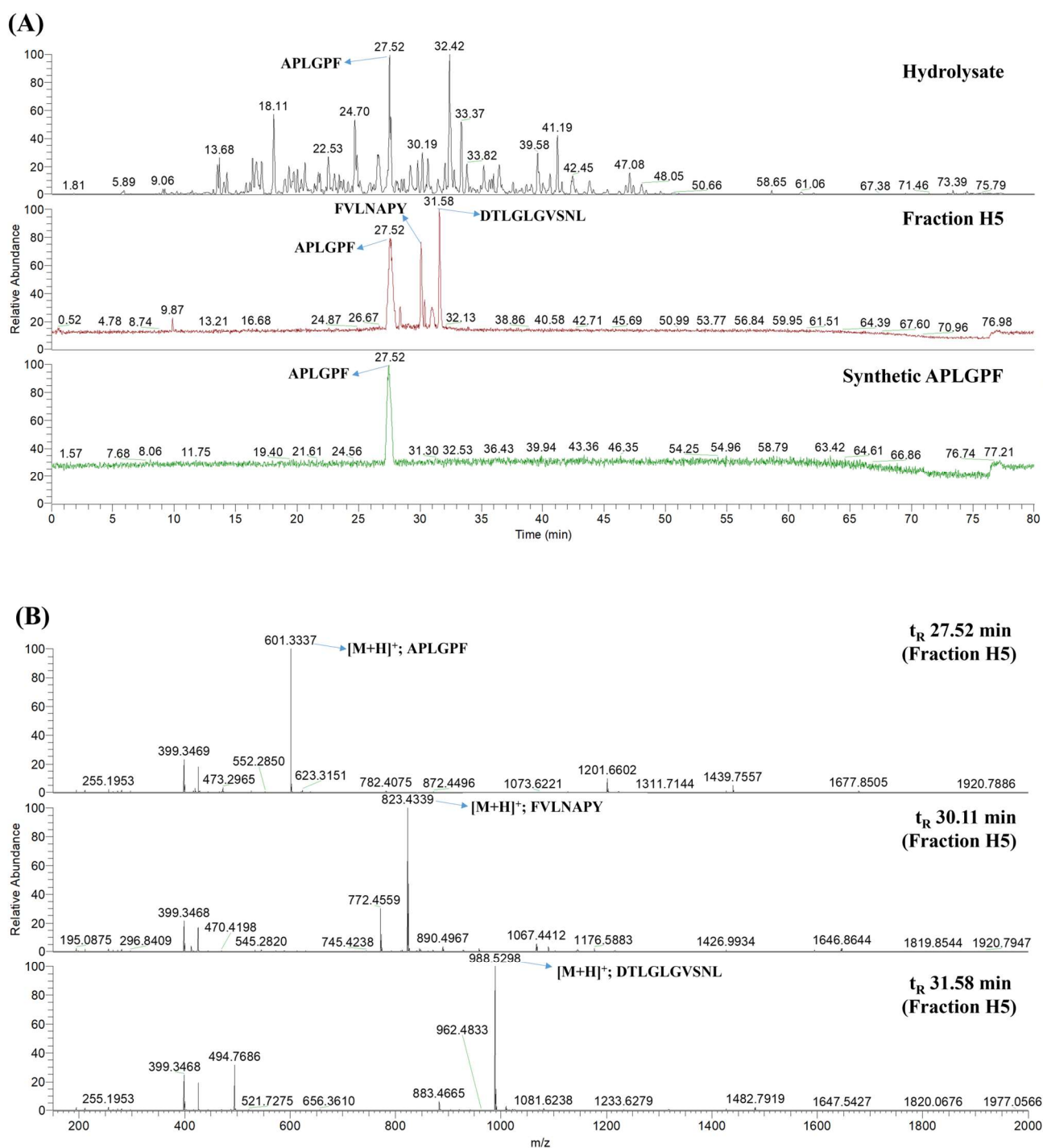


Figure 4. Cont.

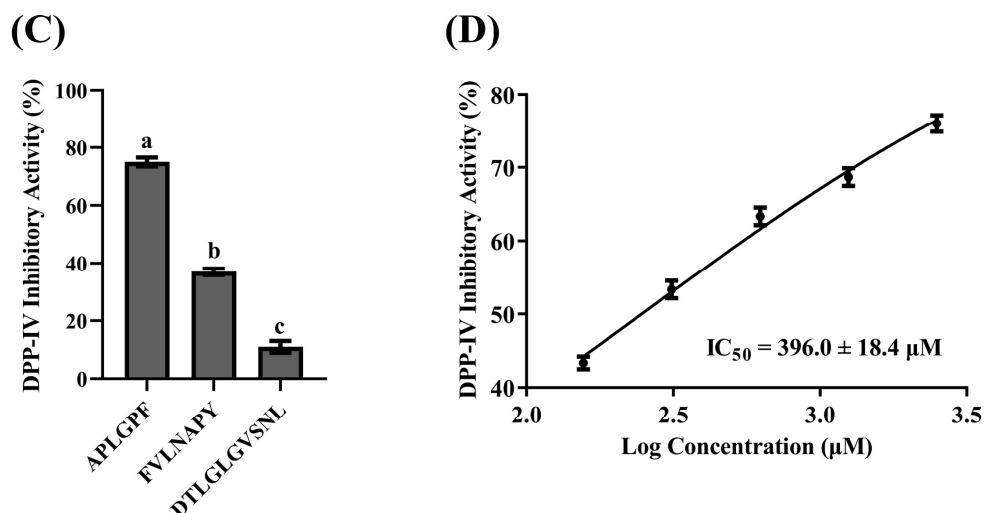


Figure 4. (A) LC-MS chromatogram of TBSP SGI hydrolysate (top panel), RP-HPLC fraction H5 (middle panel), and synthetic APLGPF (AF6). (B) MS ion spectrum confirming APLGPF (top panel, t_R 27.52 min), FVLNAPY (middle panel, t_R 30.11 min), and GTLGLGVSNL (bottom panel, t_R 31.58 min) in RP-HPLC fraction H5. (C) DPP-IV inhibitory activity of APLGPF, FVLNAPY, and DTLGLGVSNL. Values are presented as mean $\pm$ SEM ($n = 3$). Bars with different letters indicate significant differences within each group ($p < 0.05$). (D) DPP-IV IC₅₀ value of APLGPF (AF6).

3.5. Mechanism of AF6 Inhibition and Molecular Interactions with DPP-IV

The inhibitory mode of AF6 against DPP-IV was evaluated using Lineweaver–Burk analysis (Figure 5A). The plots revealed that increasing concentrations of AF6 (120 and 360 μM) altered the y -intercept without affecting the x -intercept, indicating a non-competitive inhibition pattern. This suggests that AF6 binds to a site distinct from the enzyme's active site, reducing catalytic efficiency without influencing substrate binding. Interestingly, Hung et al. [31] reported a hexapeptide containing Ala¹-Pro² at its N-terminus that also exhibited non-competitive inhibition, supporting the relevance of this structural motif in allosteric binding. To further elucidate the molecular basis of AF6–DPP-IV interactions and confirm the potential allosteric binding mode, molecular docking simulations were subsequently performed.

Molecular docking analysis demonstrated that AF6 binds outside the catalytic pocket, in a region distinct from the competitive inhibitor Diprotin A (Figure 5B). This binding mode is characteristic of allosteric inhibition, in which ligand binding induces conformational changes that impair enzyme activity without directly blocking the active site. Interaction analysis further revealed that AF6 formed stable hydrogen bonds with Arg126 and Arg358, together with additional π -interactions involving His126 and Phe357 (Figure 5C). Collectively, these intermolecular interactions, in conjunction with the kinetic data, confirm that AF6 inhibits DPP-IV through an allosteric mechanism involving residues outside the catalytic site. As a non-competitive inhibitor of DPP-IV, AF6 is likely less influenced by substrate concentration [34]. By binding at an allosteric site, AF6 may reduce the enzyme's catalytic efficiency without preventing incretin binding, which could slow incretin degradation and potentially contribute to improved postprandial glucose regulation.

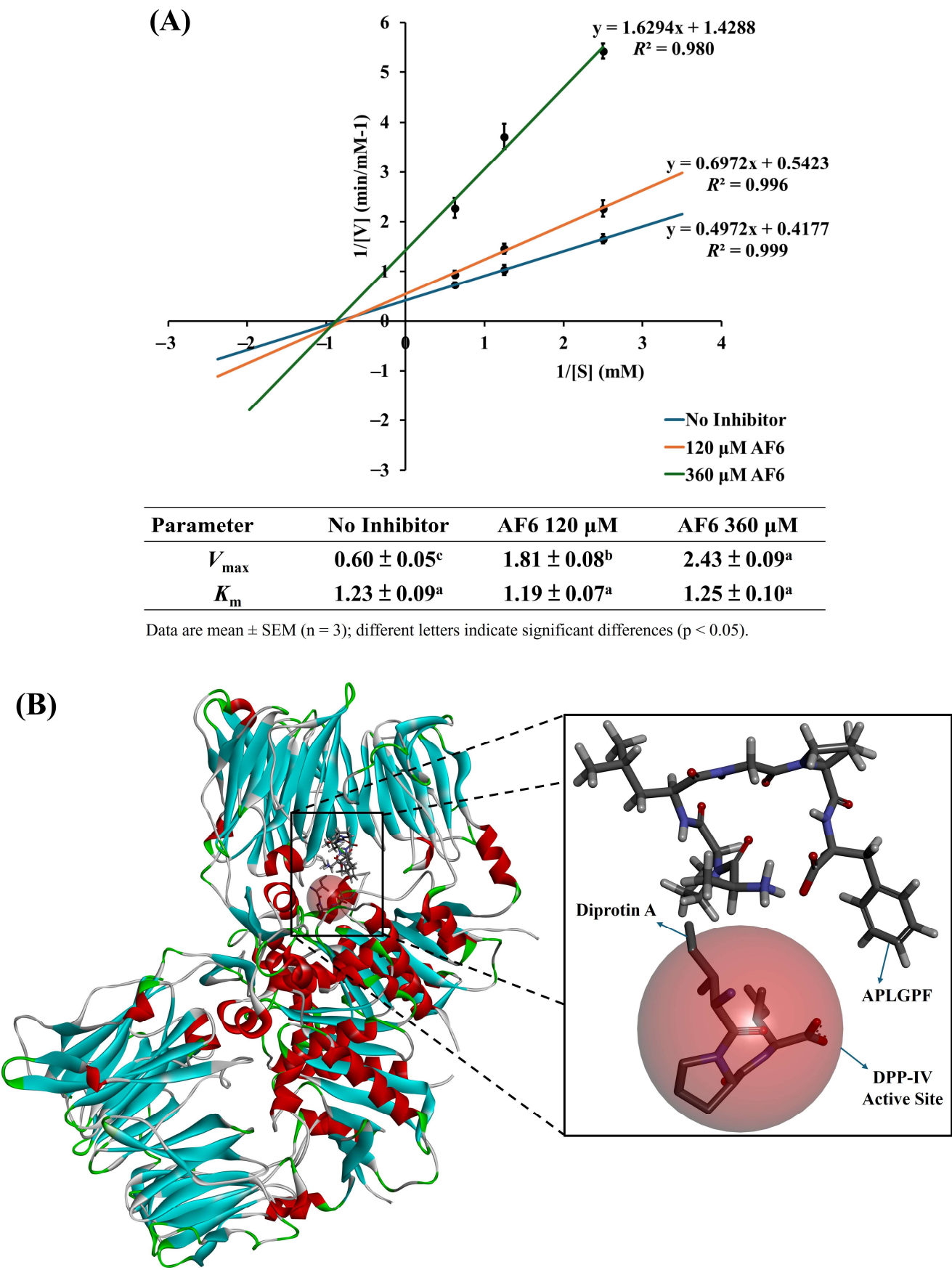


Figure 5. Cont.

(C)

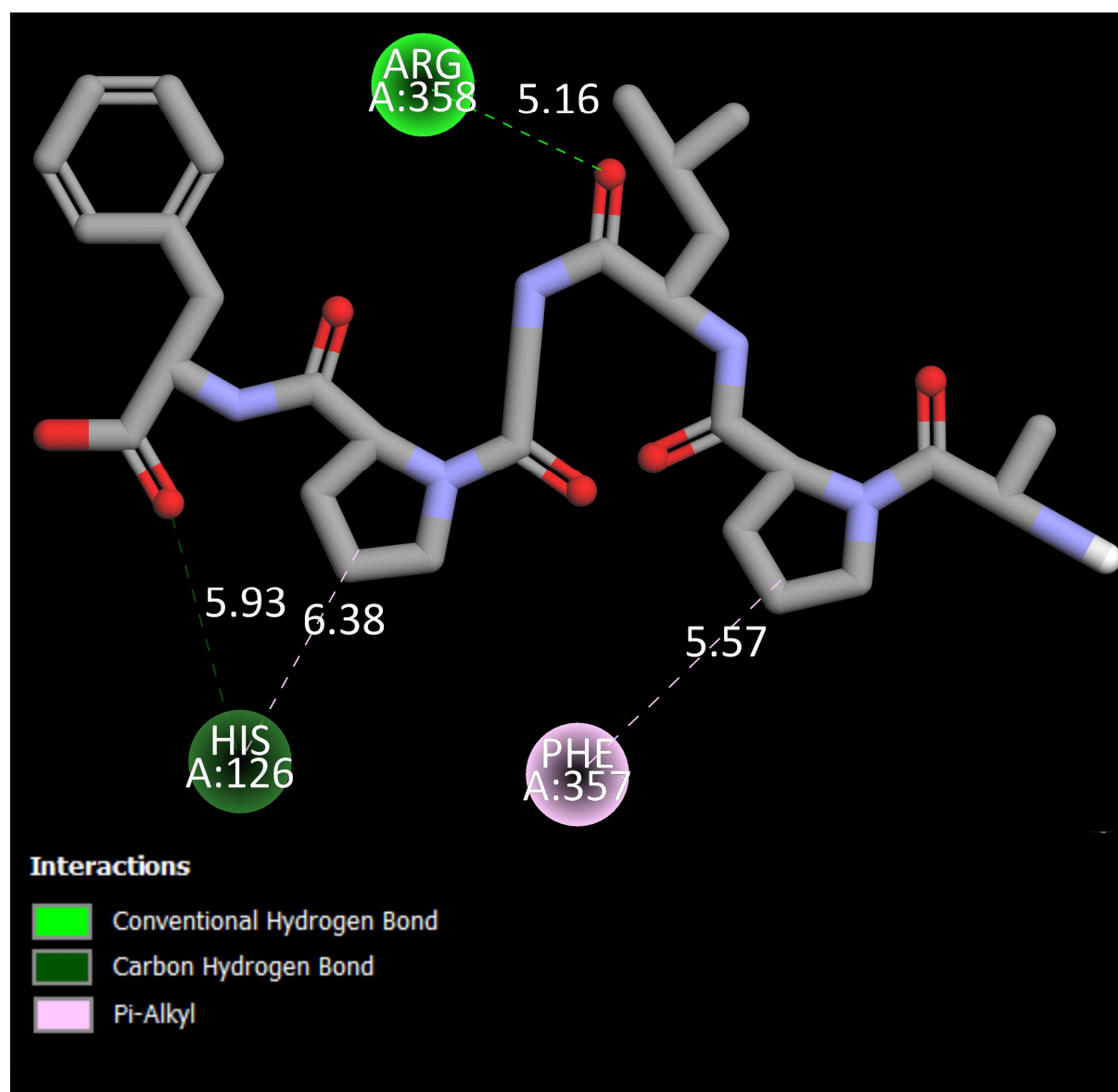


Figure 5. (A) Lineweaver–Burk plot of APLGPF (AF6) toward DPP-IV. (B) 3D visualization of the interaction between AF6 and DPP-IV. (C) 2D visualization of the interaction between AF6 and DPP-IV residues.

3.6. Stability of AF6 Toward DPP-IV and Gastrointestinal Proteases

As a peptidase, DPP-IV cleaves its natural substrates at the N-terminal dipeptides His⁷-Ala⁸ and Tyr¹-Ala² from GLP-1 and GIP, respectively [35]. Therefore, the stability of DPP-IV inhibitory peptides must be evaluated to determine whether DPP-IV alters their activity. This stability is generally classified into three functional categories: true inhibitors, real substrates, and prodrugs [24,36]. True inhibitors show no significant change in activity after pre-incubation. Real substrates exhibit reduced activity after pre-incubation, whereas prodrugs show increased activity.

To determine the interaction type and stability of AF6 toward DPP-IV, a pre-incubation assay was conducted at 2.5 mg/mL. In the regular assay (without pre-incubation) group, AF6, DPP-IV, and the GP-pNA substrate were incubated simultaneously at 37 °C for 1 h. In

the pre-incubation experiment, AF6 and DPP-IV were incubated together for 3 h before adding GP-pNA, followed by an additional 1 h incubation. As shown in Figure 6A, the inhibitory activity of AF6 remained unchanged after prolonged pre-incubation with DPP-IV, indicating that AF6 acts as a true inhibitor.

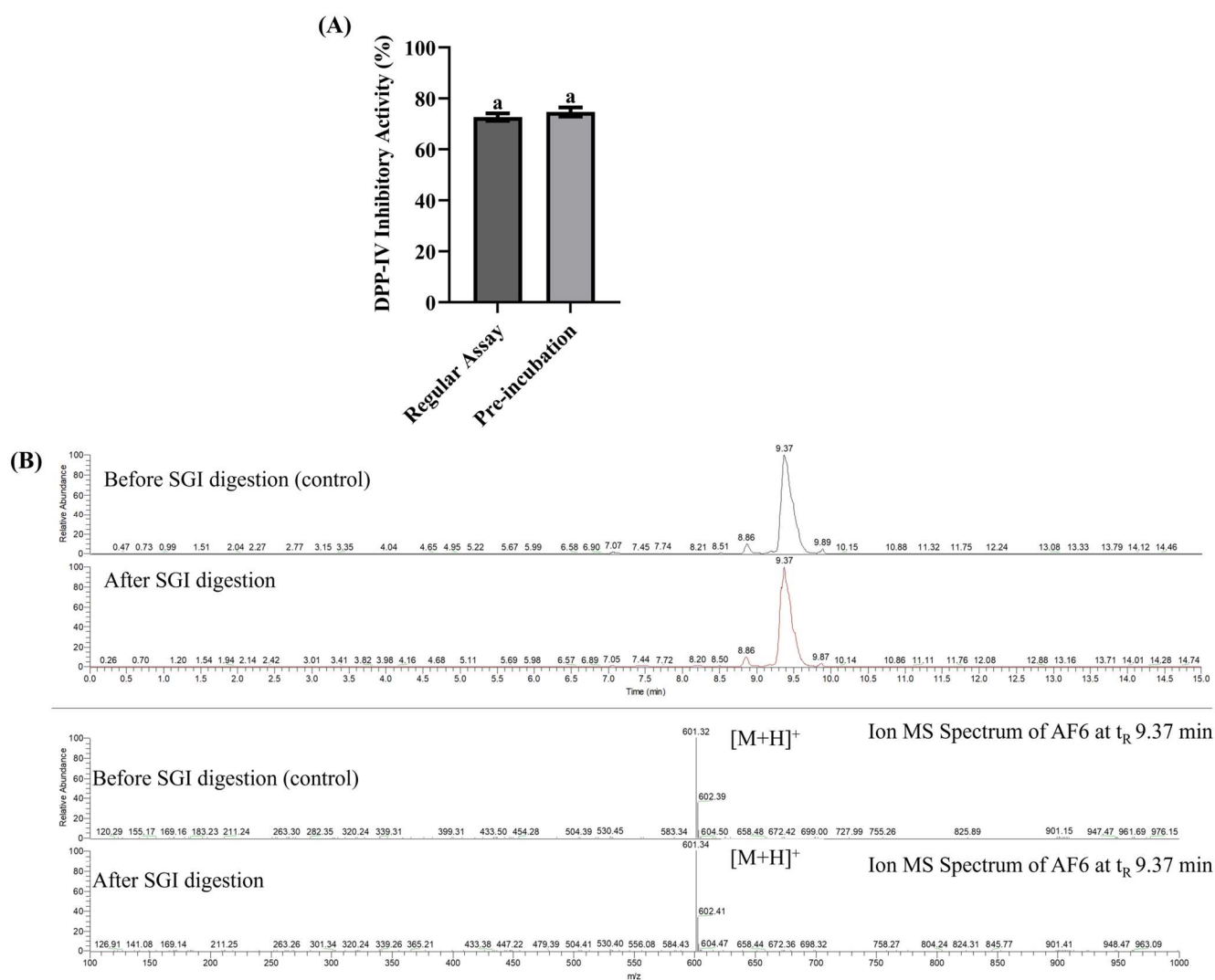


Figure 6. (A) AF6 stability against DPP-IV Pre-incubation. Values are presented as mean $\pm$ SEM ($n = 3$). Bars with different letters indicate significant differences within each group ($p < 0.05$). (B) LC-MS chromatogram of AF6 before and after SGI digestion (top panel) and ion MS spectrum of AF6 before and after SGI digestion (bottom panel).

The activity of an active peptide should remain unaltered in the presence of DPP-IV, given its abundance in the human intestine, and resistance to gastrointestinal digestion is also essential because such peptides are typically administered orally [37,38]. Therefore, the stability of AF6 toward gastrointestinal (SGI) proteases—pepsin, trypsin, and chymotrypsin—was examined, as previously described [15]. Although no reference peptides were included for direct comparison in this study, the protocol represents a general approach for assessing the resistance of peptides to enzymatic degradation during digestion. At 0.625 mg/mL, AF6 was either maintained before digestion (control) or sequentially incubated with pepsin (2 h, 37 °C), followed by trypsin and chymotrypsin (4 h, 37 °C). LC-MS/MS analysis (Figure 6B) indicated that AF6 retained a substantial degree of structural integrity after exposure to both gastric and intestinal phases, suggesting potential

resistance to proteolytic degradation under simulated human digestive conditions. The low molecular mass of AF6 (Ala¹-Pro²-Leu³-Gly⁴-Pro⁵-Phe⁶), together with its proline residues, may significantly enhance its resistance to gastrointestinal protease digestion [31]. Collectively, the ability of AF6, a novel DPP-IV inhibitory hexapeptide derived from tree bean seeds, to resist degradation by both DPP-IV and gastrointestinal proteases suggests that it may retain bioactivity following oral ingestion. This characteristic enhances its potential as an orally active antidiabetic peptide. While further comparative studies are needed to fully contextualize the stability of AF6 relative to other peptides, the present findings support its promise as a candidate for nutraceutical development aimed at postprandial glycemic control.

4. Conclusions

This study reports the first identification of a DPP-IV inhibitory peptide from *Parkia timoriana* seeds. SGI hydrolysate and sequential fractionation yielded APLGPF (AF6), a hexapeptide with favorable inhibitory activity (DPP-IV IC₅₀ = 396 ± 18 µM). Enzyme kinetics and docking confirmed an allosteric, non-competitive inhibition mode, while stability assays demonstrated resistance to both DPP-IV and gastrointestinal proteases. These properties highlight AF6 as a stable, orally relevant antidiabetic peptide. Beyond providing scientific evidence for the bioactivity of an underutilized legume, this work positions *P. timoriana* as a potential source for functional foods or nutraceuticals targeting type 2 diabetes, contributing to global health efforts in line with Sustainable Development Goal 3. Further studies on the bioavailability, absorption, and in vivo efficacy of AF6 will be essential to validate its physiological activity and support its future development as a nutraceutical candidate for diabetes management.

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