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Exploring dipeptidyl peptidase-IV inhibitory peptides from Tartary Buckwheat protein: A study of hydrolysis, fractionation, and molecular interactions

Weijing Wu[1,2] | **Libo Wang**[3] | **Ju Qiu**[4] | **Ziyuan Zhuang**[1] | **Fei Qin**[1] | **Qianglai Tan**[1] | **Yanling Wang**[1] | **Lanlan Wu**[1]

[1]Xiamen Medical College, Jimei district, Xiamen, China

[2]Engineering Research Center of Natural Cosmeceuticals College of Fujian Province, Xiamen, China

[3]College of Food and Bioengineering, Henan University of Science and Technology, Luoyang, China

[4]Key Laboratory of Precision Nutrition and Food Quality, Department of Nutrition and Health, China Agricultural University, Beijing, China

Correspondence
Lanlan Wu, Xiamen Medical College, No. 1999 Guankou Middle Road, Jimei District, Xiamen, Fujian, 361023, China.
Email: hanslan@sina.cn

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Abstract: Tartary buckwheat, a protein-rich pseudocereal with anti-diabetic effects, has not yet been fully explored as a source of dipeptidyl peptidase-IV (DPP-IV) inhibitory peptides. This study aims to discover novel DPP-IV inhibitory peptides from tartary buckwheat protein (TBP). Five hydrolysis methods were employed, with simulated gastrointestinal digestion (SGID) releasing the most active DPP-IV inhibitory hydrolysates, showing both the highest degree of hydrolysis (22.66 ± 1.12%) and inhibition activity (41.81 ± 1.52% at 1.25 mg/mL). In addition, ultrafiltration enriched the <3 kDa fraction with the highest inhibitory rate, and further purification using reverse-phase high-performance liquid chromatography concentrated the DPP-IV inhibitory peptides in the first fraction (F1). Nano liquid chromatography-tandem mass spectrometry analysis identified 10 new peptides in F1, among which the peptide Leu-His-Ile-Val-Gly-Pro-Asp-Lys (LHIVGPDK) exhibited the strongest inhibitory effect, with an IC_{50} value of 1.61 mM. Kinetic studies revealed that LHIVGPDK acts as a mixed-type inhibitor, and molecular docking indicated that it inhibits DPP-IV by forming stable complexes through five types of interactions, with hydrogen bonds playing a key role. This study underscores TBP's DPP-IV inhibitory potential and anti-diabetic properties, reinforcing the value of tartary buckwheat as a beneficial food for diabetes management.

KEYWORDS
DPP-IV, sitagliptin, tartary buckwheat peptides

Practical Application: This research identified new peptides from Tartary buckwheat protein that effectively inhibit DPP-IV, an enzyme associated with diabetes management. These findings suggest potential applications in developing functional foods to help control hypoglycemia.

1 | INTRODUCTION

Diabetes mellitus, a chronic metabolic disorder caused by insufficient insulin and/or insulin resistance, poses a significant public health challenge (Sun et al., 2022). Inhibiting dipeptidyl peptidase-IV (DPP-IV) is a therapeutic strategy to enhance insulin secretion and stabilize blood glucose by preventing the degradation of crucial peptides such as glucagon-like peptide-1 and glucose-dependent insulinotropic peptide (Deacon, 2019). Despite their effectiveness, long-term concerns about DPP-IV inhibitors such as sitagliptin remain. Thus, bioactive peptides from food are being explored as alternatives to DPP-IV inhibitors (Zhang et al., 2024). Managing staple food intake is crucial for diabetes control due to its high starch content. Grains and pseudocereals, as key protein sources in the daily diet, may release DPP-IV inhibitory peptides that help to simultaneously modulate blood glucose released from their own starch. Thus, studying these peptides in staple foods can help in selecting healthier options for diabetes management. Protein hydrolysates from common grains, including rice (Hatanaka et al., 2015), wheat (Ding et al., 2022), and oat (Mu et al., 2024), have demonstrated DPP-IV inhibition, with new peptides identified. Alternatively, pseudocereals such as quinoa and millet may offer superior conditions for generating DPP-IV inhibitory peptides compared to common grains, due to their diverse peptide profiles (Usman et al., 2022). Native quinoa and millet proteins lacked DPP-IV inhibitory effects, but hydrolysis significantly boosted their potency, identifying several new DPP-IV inhibitory peptides (Gu et al., 2021; Nongonierma et al., 2015; Vilcacundo et al., 2017; You et al., 2022).

Among pseudocereals, tartary buckwheat (*Fagopyrum tataricum*), valued for its nutritional richness and resilience to harsh climates, has been historically cultivated in Asia and Central Europe and is now gaining global recognition as a functional food (Ruan et al., 2022). Tartary buckwheat contains 9.06% to 14.88% high-quality proteins with a balanced amino acid composition (Bhinder et al., 2020). Substituting white rice with tartary buckwheat can enhance dietary protein levels (Qiu et al., 2016), providing greater potential for peptide release. As an under-researched pseudocereal, tartary buckwheat's unique protein composition may offer a new source of DPP-IV inhibitory bioactive peptides. Tartary buckwheat protein (TBP) hydrolysates exhibit various biological activities, particularly in antioxidant effects and renin inhibition (Li et al., 2024, 2023; Luo et al., 2020; Xiao et al., 2023; Yang et al., 2023; Zhou et al., 2020; Zhu, 2021). However, most TBP-derived peptides come from albumin or globulins, while the potential of other fractions such as prolamin and glutenin (Guo & Yao, 2006) remains unexplored. Tartary buckwheat is a functional staple food, particularly effective in controlling hypoglycemia (Qiu et al., 2016; Ruan et al., 2022). However, aside from commonly studied bioactive compounds in tartary buckwheat, such as flavonoids (Ruan et al., 2022), research on TBP-derived peptides with hypoglycemic effects, especially those targeting DPP-IV inhibition, remains limited.

Therefore, this research aims to identify novel DPP-IV inhibitory peptides from whole TBP, contributing to the understanding of tartary buckwheat's hypoglycemic activity. The study compared five hydrolysis methods to maximize the release of DPP-IV inhibitory peptides. Ultrafiltration and reverse-phase high-performance liquid chromatography (RP-HPLC) were employed to purify peptides, with their sequences identified via nano liquid chromatography-tandem mass (nano LC-MS/MS). Molecular docking and enzymatic kinetics were used to explore the structure–activity relationships of the newly identified DPP-IV inhibitory peptide from TBP.

2 | MATERIALS AND METHODS

2.1 | Materials and reagents

Tartary buckwheat flour was provided by Luoji Mountain Company. Bovine serum albumin (BSA) was provided by Beijing Biodragon Immunotechnologies Co., Ltd. 2,2′-Biquinoline-4,4′-dicarboxylic acid disodium salt (BCA), Coomassie Blue-R250, and trypsin (P110505, ≥2100 U/g) were purchased from Aladdin Bio-Chem Technology Co., Ltd. Alcalase 2.4 U/g (P4860), dipeptidyl peptidase IV human (DPP-IV, D4943, ≥10 U/mg), and Gly-Pro-pNA (Gly-Pro-p-Nitroaniline, G0513) were obtained from Sigma-Aldrich. Papain (S10011, ≥800 U/mg), Neutrase (S10013, ≥100 U/mg), and Flavorzyme (S10153, ≥20 U/mg) were sourced from Yuanye Bio-Technology Co., Ltd. Pepsin ≥250 U/mg (403H021) and L-Phenylalanine standard were purchased from Solarbio Life Sciences. Synthetic peptides were obtained by Sangon Biotech (Shanghai) Co., Ltd. All other reagents were of analytical grade.

2.2 | Methods

2.2.1 | Preparation of whole TBP isolates

The isolation of TBP was adapted from the method established in previous research (Wu et al., 2021). Tartary buckwheat flour was first defatted using petroleum ether in a 1:5 (w/v) ratio, stirred at 600 r/m for 1 h at room temperature. The defatted flour was then vacuum filtered and

air-dried. Next, the defatted flour was mixed with deionized water at a 1:10 (w/v) ratio, and the pH was gradually adjusted to 10.0 using 1 M NaOH. The mixture was stirred for 1 h while maintaining the pH at 10.0, after which it was centrifugated at 6000 r/m for 5 min. The pH of the resulting supernatant was then adjusted to 4.50 using 1 M HCl to precipitate the TBP. The precipitated TBP was collected by centrifugation at 5500 r/m, re-dissolved in deionized water, and then the pH was adjusted to 7.0 using 1 M NaOH. This solution was dialyzed for 24 h to remove salts and then freeze-dried. The protein content in TBP isolates, determined by the Coomassie Blue method, was 84.39 ± 0.16% (w/w).

2.2.2 | Preparation of TBP hydrolysates

The hydrolysis of TBP was conducted using five different methods, with modifications based on previous studies (He et al., 2023; Zhang et al., 2022). TBP isolates were dissolved in deionized water at a concentration of 2% (w/v), and the pH was gradually adjusted to 7.0 using 1 M NaOH. The solution was stirred at room temperature for 1 h to ensure complete dissolution. Subsequently, the protein solution was heated at 95°C for 10 min to induce protein denaturation, increasing susceptibility to enzymatic hydrolysis, and then cooled to the optimal temperature for different enzymes. The TBP solution was hydrolyzed by adding four individual enzymes at a ratio of 4% enzyme/substrate (E/S) for 3 h, while maintaining the following conditions: Neutrase at 45°C and pH 8.0, Flavorzyme at 37°C and pH 7.5, Papain at 55°C and pH 7.5, and Alcalase at 55°C and pH 8.0. In vitro gastrointestinal digestion (SGID) was simulated using pepsin and pancreatin, as described in a previous study (Wu et al., 2021). Gastric digestion with pepsin was performed at 37°C and pH 2.0 for 1 h, followed by intestinal digestion with pancreatin at 37°C and pH 8.0 for additional 2 h, both using 4% E/S. The pH of the hydrolysis system was maintained at the optimal level for each enzyme using 1 M NaOH and 1 M HCl throughout the entire hydrolysis process. Samples were collected at 0, 1, and 3 h, heated in boiling water for 10 min to deactivate the enzymes, and then centrifuged at 10,000 r/m for 10 min to obtain the supernatant containing the hydrolysis products.

2.2.3 | Determination of degree of hydrolysis

The degree of hydrolysis (DH) was determined using the ortho-phthalaldehyde (OPA) method (Zhang et al., 2016). In this method, 50 μ-L of appropriately diluted supernatant was mixed with 3 mL of OPA reagent, which consisted of 100 mM sodium tetraborate, 0.01% sodium dodecyl sulfate, 0.05 mg/mL OPA, and 0.2% β-mercaptoethanol (prepared fresh as needed). The reaction was conducted at 37°C for 2 min, protected from light, and the absorbance was measured at 340 nm. A standard curve was prepared by plotting the absorbance against the concentration of L-phenylalanine standards ranging from 0 to 7.58 mM. The DH was then calculated using the following formula:

$$\mathrm{DH}\% = \frac{(N - N_0)}{C} \times \frac{1}{\mathrm{h_{tot}}}, \quad (1)$$

where C represents the concentration of TBP (g/mL); N denotes the concentration of amino nitrogen in the TBP hydrolysate (mmol/mL); N_0 indicates the concentration of amino nitrogen in the unhydrolyzed TBP solution (mmol/mL); and h_{tot} is the molar number of peptide bonds in the protein, which is 7.72 mmol/g.

2.2.4 | Determination of peptide concentration by the BCA assay

Following the procedure described (Vilcacundo et al., 2017), 20 μL of the sample was mixed with 200 μL of BCA reagent and incubated at 60°C for 15 min. After incubation, the absorbance was measured at 570 nm. To quantify peptide concentrations, a standard curve was generated using BSA standard solutions with concentrations ranging from 0 to 1 mg/mL.

2.2.5 | In vitro DPP-IV inhibition test

The in vitro DPP-IV inhibition activity of TBP and its hydrolysates was evaluated using Gly-Pro-pNA as a substrate, as described in a previous study with modifications (Wang et al., 2015). The peptide content in different fractions was measured using the BCA assay, as detailed in Section 2.2.4. Subsequently, 25 μL of each sample solution at designed concentrations was prepared using 100 mM Tris-HCl solution (pH 8.0) and mixed with 25 μL of Gly-Pro-pNA at 1 mM in a 96-well plate. The mixture was incubated at 37°C for 5 min with shaking. The reaction was activated by adding 50 μL of DPP-IV solution (8 U/L). Incubation continued at 37°C for 30 min. Absorbance at 405 nm was recorded at the beginning (0 min) and after 30 min of incubation. The DPP-IV inhibition activity was calculated using the following formula formatting,

with Diprotin used as a positive control to confirm assay performance.

$$\text{DPP}-\text{IVInhibition}\ \% = \frac{\left[\left(A_{\text{negative 30 min}} - A_{\text{negative 0 min}}\right) - \left(A_{\text{sample 30 min}} - A_{\text{sample 0 min}}\right)\right]}{\left(A_{\text{negative 30 min}} - A_{\text{negative 0 min}}\right)} \times 100\%, \quad (2)$$

where A_{negative} is the absorbance of the 100 mM Tris-HCl (pH 8.0) solution replaced with samples.

2.2.6 | Ultrafiltration

To separate the TBP hydrolysates, ultrafiltration was employed as previously described (Hong et al., 2020). The samples were subjected to centrifugation at 8000 g and 4°C for 30 min using a 10 kDa molecular weight cutoff filter. The resulting filtrate was then transferred to a 3 kDa cut-off filter and centrifuged again under the same conditions (8000 g, 4°C, 30 min) to further separate the peptide mixtures. This process yielded three distinct fractions based on molecular weight: <3 kDa, 3 kDa-10 kDa, and >10 kDa. Each fraction was collected, and the peptide concentrations were determined using the BCA assay described in Section 2.2.4, while the DPP-IV inhibition activity of these fractions was assessed at the same concentrations following the method described in Section 2.2.5.

2.2.7 | Reverse-phase high-performance liquid chromatography

The <3 kDa fraction, which demonstrated the most significant DPP-IV inhibition, was subjected to further separation using RP-HPLC (Zhang et al., 2016) with modifications. An e2695 HPLC system (Waters) was employed, equipped with an Acclaim 120, C18 column (250 × 4.6 mm, 5 μm, No. 059149) from Thermo Fisher Scientific. The mobile phases consisted of solvent A: ultrapure water with 0.1% trifluoroacetic acid (TFA), and solvent B: acetonitrile with 0.1% TFA. The gradient elution program was as follows: 0 min with 5% solvent B, 5 min with 10% solvent B, 22 min with 22.8% solvent B, 24 min with 60% solvent B, 25 min with 5% solvent B, and 30 min with 5% solvent B. The column was maintained at room temperature, and the flow rate was set to 1 mL/min. Fractions with absorbance peaks at 220 nm were collected and designated as F1, F2, F3, F4, F5, and F6. Each fraction was concentrated by rotary evaporation, and peptide concentrations were determined using the BCA assay (Section 2.2.4). The DPP-IV inhibition activity of each fraction was evaluated at a consistent peptide concentration (Section 2.2.5).

2.2.8 | Identification of peptide sequences by nano LC-MS/MS

Nano LC-MS/MS was employed to identify the sequences of DPP-IV inhibitory peptides, following a modified method (Zhou et al., 2024). The analysis utilized two elution solvents: solvent A (98% water, 2% acetonitrile, 0.1% formic acid) and solvent B (98% acetonitrile, 2% water, 0.1% formic acid). A C18 nano Acquity column (10K psi, 130Å, 100 μm × 100 mm, 1.7 μm, Waters) was used for sample separation, with a flow rate set at 0.3 μL/min. The gradient elution program was as follows: at 0 min, 98% solvent A; 5 min, 90% solvent A; 85 min, 65% solvent A; 105 min, 35% solvent A; 110 min, 5% solvent A; and 120 min, 5% solvent A. Q Exactive mass spectrometry parameters (Thermo Fisher Scientific) included a spray voltage of 2.1 kV, sheath gas at 50 arb, auxiliary gas at 13 arb, capillary temperature at 300°C, S-lens RF at 60, and a scan range of 350–2000 m/z with resolutions of 70,000 for the primary and 17,500 for the secondary mass spectrometry. The MS data were processed using the Thermo Scientific Xcalibur platform. Peptide sequences of TBP were confirmed using the Proteome Discoverer software and the BLAST tool.

2.2.9 | Enzymatic kinetics analysis

Based on the peptide sequences identified in Section 2.2.8, 10 peptides were synthesized using solid-phase synthesis technology by Shenggong Bioengineering (Shanghai) Co., Ltd. The DPP-IV inhibition activity of these synthesized peptides was tested at equivalent concentration.

The median inhibitory concentration (IC_{50}) of LHIVGPDK was determined from the plot of DPP-IV inhibition against the final concentrations of the sample used. Next, the Lineweaver-Burk method (Gu et al., 2021) was then employed to analyze the inhibition type of the active peptide LHIVGPDK. The concentrations of active peptides were adjusted to IC_{50}, $IC_{50}/2$, and 0 M, while Gly-Pro-pNA varied at 0.25, 0.5, 1.0, 1.5, and 3.0 mM. The initial velocity of the reaction was determined by

measuring the change in absorbance over a 30-min period.

2.2.10 | Molecular docking analysis of peptide with DPP-IV

Molecular docking techniques were utilized to investigate the binding conformation of active peptides with DPP-IV (Zan et al., 2023). The three-dimensional crystal structure of the DPP-IV enzyme (PDB code: 5J3J) was retrieved from the Protein Data Bank. Before docking, co-crystallized ligands and water molecules were removed using the PyMOL 2.4 software (Schrodinger, LLC). The structure of the active peptide LHIVGPDK was drawn and optimized using ChemDraw 19.0 software. AutoDock 4.2.6, which employs the Lamarckian genetic algorithm, was used to explore potential interactions between the peptide and the enzyme, focusing on the catalytic site of DPP-IV designated as the docking target. The model demonstrating the lowest binding energy was selected as the optimal binding conformation. The resulting peptide-enzyme complexes were visualized and further analyzed using PyMOL and Discovery Studio 4.5 software.

2.2.11 | Statistical analysis

The experiments were conducted at least twice, and data were expressed as mean ± standard deviation (mean ± SD). Statistical analysis was conducted using one-way analysis of variance, followed by Duncan's multiple range test in SPSS, to determine significant differences among different groups ($p < 0.05$).

3 | RESULTS AND DISCUSSION

3.1 | DH and DPP-IV inhibitory activity of TBP with different protease hydrolyses

Different proteases have different cleavage sites, resulting in varied abilities to produce hydrolysis products. The DH is an effective indicator for evaluating the extent of peptide bond cleavage during protein hydrolysis. As shown in Figure 1a, different enzymes significantly increased the DH of TBP over 3 h of hydrolysis ranging from 1.69 ± 0.37% (Papain) to 22.66 ± 1.12% (SGID). The DH of TBP through SGID was significantly higher than other methods ($p<0.05$), with Alcalase following at 12.79 ± 0.23%. Notably, trypsin digestion markedly increased the DH during gastric digestion, from 2.93 ± 0.21% to 22.66 ± 1.12%. The low DH in the first hour of gastric digestion may be attributed to the low solubility of TBP, which affects pepsin's efficiency. In the subsequent 2 h, trypsin, with its numerous cleavage sites, effectively enhances hydrolysis efficiency. The similar DH observed in TBP hydrolysis is consistent with previous findings, where enzymatic hydrolysis of TBP with trypsin achieved a 24% DH after 9 h of hydrolysis (Tang et al., 2008). In comparison, Neutrase, Flavorzyme, and apain exhibited relatively lower DH values, ranging from 1.69 ± 0.37% to 9.58 ± 0.24%. Extending the hydrolysis time did not significantly increase the DH for these three enzymes, highlighting the significant impact of enzyme type on the DH of TBP. Therefore, SGID, with the highest DH, may be more effective in releasing DPP-IV inhibitory peptides.

The TBP itself exhibited no DPP-IV inhibition (Figure 2a). As shown in Figure 1b, enzymatic hydrolysis for 3 h significantly enhanced the DPP-IV inhibitory activity of TBP ranging from 17.17 ± 0.04% (papain) to 41.81 ± 1.52% (SGID; $p<0.05$). This finding aligns with previous studies on quinoa and millet proteins, which also showed minimal DPP-IV inhibitory effects in their native forms but demonstrated a significant increase in inhibitory activity following hydrolysis (Gu et al., 2021; Guo et al., 2020; Nongonierma et al., 2015; Vilcacundo et al., 2017; You et al., 2022). With increasing hydrolysis time, the DPP-IV inhibition of TBP hydrolysates by SGID also increased, particularly with trypsin digestion, which significantly raised the DPP-IV inhibitory rate from 22.23 ± 0.04% to 41.81 ± 1.52% at a final concentration of 1.25 mg/mL ($p<0.05$). This trend aligns with the DH results (Figure 1a), indicating that enzymatic hydrolysis releases peptides from TBP, thereby enhancing the DPP-IV inhibition rate of the hydrolysates. Similar to the SGID process observed with quinoa protein hydrolysis (Vilcacundo et al., 2017), intestinal digestion of buckwheat protein released DPP-IV inhibitory peptides more effectively than gastric digestion. Additionally, hydrolysis with papain, which had the lowest DH, resulted in the lowest DPP-IV inhibition rate. In contrast, the Flavorzyme, Alcalase, and Neutrase hydrolysates had relatively higher DH values compared to papain exhibited in significantly higher DPP-IV inhibitory effects ($p<0.05$). Although TBP hydrolysates digested by Alcalase and Neutrase showed similar DPP-IV inhibition rates after 3 h of hydrolysis compared to the SGID process ($p>0.05$), SGID was more effective in obtaining DPP-IV inhibitory peptides, as indicated by a highest DH and greater release of bioactive peptides. Therefore, the SGID hydrolysate, obtained after 3 h, was selected for subsequent experiments due to its superior efficiency in promoting peptide release and DPP-IV inhibition. This observation is consistent with previous research suggesting SGID as an effective method for releasing DPP-IV inhibitory peptides

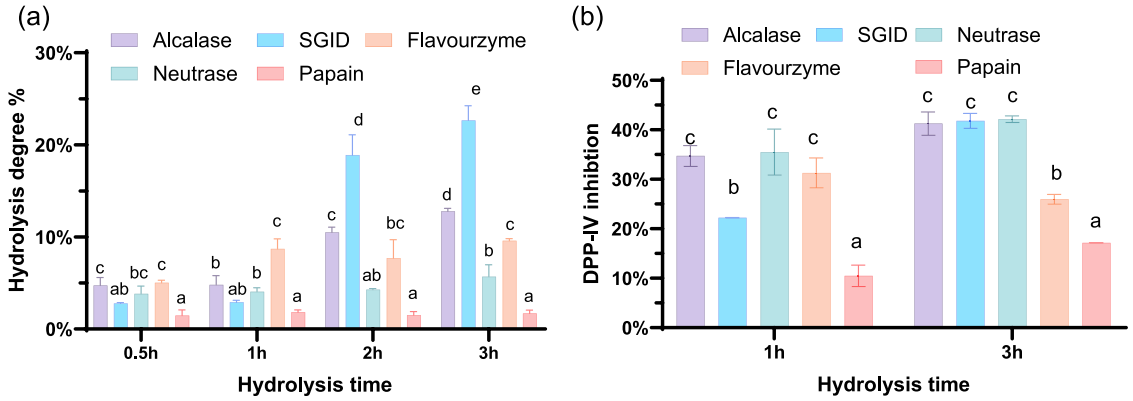

FIGURE 1 Effects of different enzymes and hydrolysis time on (a) degree of hydrolysis and (b) dipeptidyl peptidase-IV (DPP-IV) inhibitory activities of tartary buckwheat protein (TBP) hydrolysates. The DPP-IV inhibition was tested at a final concentration of 1.25 mg/mL. Different letters indicate significant differences among different groups at the same time point ($p < 0.05$). SGID, simulated gastrointestinal digestion.

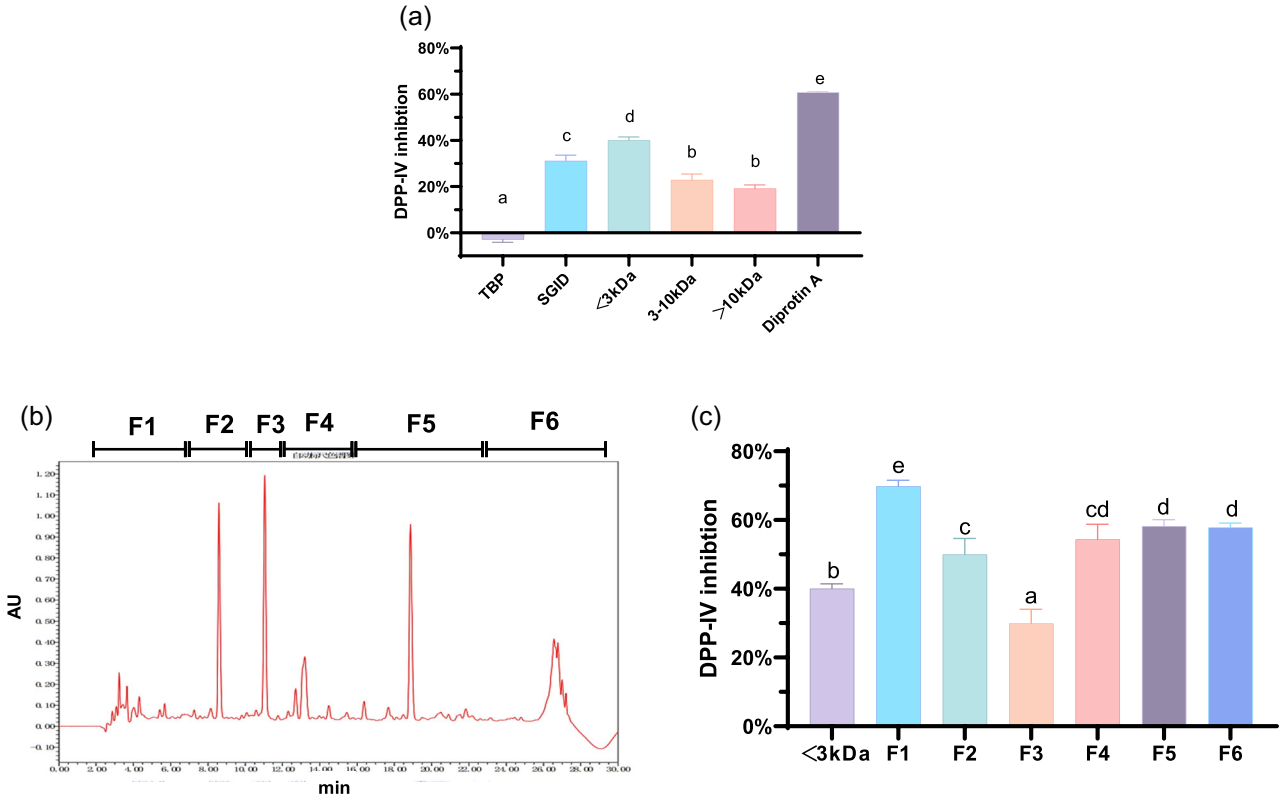

FIGURE 2 (a) Dipeptidyl peptidase-IV (DPP-IV) inhibition of tartary buckwheat protein (TBP), its simulated gastrointestinal digestion (SGID) hydrolysates, and ultrafiltration fractions; (b) reverse-phase high-performance liquid chromatography (RP-HPLC) elution profile of hydrolysates from SGID; (C) DPP-IV inhibition of peptide fractions separated by RP-HPLC. The DPP-IV inhibition was tested at a final concentration of 1.25 mg/mL. Different letters indicate significant differences among different fractions ($p < 0.05$).

(Fleury et al., 2022; Theysgeur et al., 2021; Zamudio et al., 2022). Future studies could explore combining multiple enzymes to enhance the production of DPP-IV inhibitory peptides (He et al., 2023; Ramírez Fuentes et al., 2021). Moreover, using different kinds of enzymes may release additional DPP-IV inhibitory peptides. For example, ginger protease has been shown to release DPP-IV peptides from wheat gluten more effectively than

previously reported (Ding et al., 2022; Nongonierma et al., 2017).

3.2 | Purification of DPP-IV inhibitory peptides

As shown in Figure 2a, among the three ultrafiltration fractions, the <3 kDa fraction exhibited the greatest DPP-IV inhibition compared to the 3 to 10 kDa and >10 kDa fractions ($p<0.05$). The <3 kDa fraction further enhanced DPP-IV inhibition rate, exceeding the effect of the parent SGID hydrolysate ($p<0.05$). Additionally, the positive control, Diprotin A, exhibited outstanding DPP-IV inhibitory effects. This result is consistent with literature reports that smaller molecular weight peptides have a greater inhibitory effect on DPP-IV compared to larger peptides (Gu et al., 2021; Hong et al., 2020; Li-Chan et al., 2012; Ramírez Fuentes et al., 2021; Vilcacundo et al., 2017; Y. Zhang et al., 2016), demonstrating that ultrafiltration is an effective method for concentrating these inhibitory peptides.

Based on hydrophobicity, the <3 kDa fraction was further separated using RP-HPLC, resulting in six HPLC fractions labeled F1–F6, as shown in Figure 2b. All RP-HPLC separated fractions, except for F3, exhibited significantly higher DPP-IV inhibitory activity compared to the <3 kDa fraction ($p<0.05$; Figure 2c). Among them, F1 showed the highest DPP-IV inhibition, reaching 69.83 ± 1.74% at 1.25 mg/mL. This result indicates that chromatographic separation further enriched the peptides with DPP-IV inhibitory activity. Consistent with previous studies, the low hydrophobicity fractions demonstrated higher DPP-IV inhibition (Hong et al., 2020; Li-Chan et al., 2012; Y. Zhang et al., 2016). Peptides with low DPP-IV IC_{50} values were typically, though not universally, hydrophobic (Nongonierma et al., 2014). Therefore, F1 was collected for subsequent amino acid sequence analysis.

3.3 | Novel DPP-IV inhibitory peptides identified by nano LC-MS/MS

Nano LC-MS/MS was used to identify the amino acid sequences of peptides in F1. As shown in Table 1, 10 new peptides were identified in the F1, with molecular weights ranging from 728.85 to 1603.52 g/mol and peptide lengths varying from 6 to 14 amino acids. The MS figure is provided in Figure S1. Additionally, these 10 newly identified peptides, containing fragments with potential DPP-IV inhibitory activity as sourced from the BIOPEP database, are listed in Table S1, highlighting their DPP-IV inhibitory potential.

TABLE 1 Ten new peptides identified from the F1 fraction.

No.	Sequence	Molecular weight (g/mol)	Length
1	RGENDQRG	930.92	8
2	QmPEPK	728.85	6
3	ESESQESSRGDQHQ	1603.52	14
4	RLSIRPP	838.01	7
5	DVDRETIS	933.96	8
6	LHIVGPDK	878.02	8
7	NAIIGPR	739.86	7
8	YKNAIIGPR	1031.21	9
9	AIGDIPNLI	925.08	9
10	KNNDNAITSPI	1186.27	11

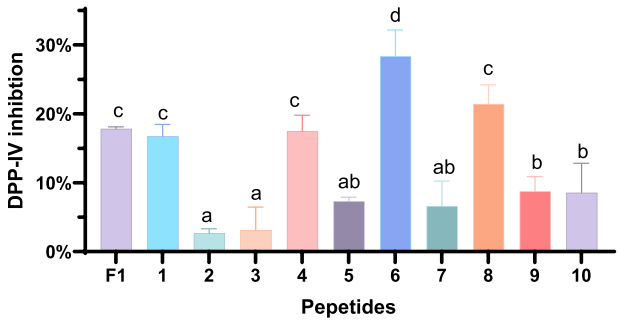

FIGURE 3 Dipeptidyl peptidase-IV (DPP-IV) inhibition of 10 synthetic peptides at a final concentration of 0.5 mg/mL. Different letters indicate significant differences among the peptides ($p < 0.05$).

To validate the identified novel peptides, their actual DPP-IV inhibitory effects were confirmed using the synthetic peptides, as illustrated in Figure 3. Among them, LHIVGPDK (No. 6) exhibited the highest inhibitory effects against DPP-IV compared to the other peptides and was significantly higher than the F1 at final concentration of 0.5 mg/mL ($p<0.05$). Additionally, the peptides RGENDQRG (No. 1), RLSIRPP (No. 4), and YKNAIIGPR (No. 8) also demonstrated relatively higher DPP-IV inhibitory activity than the other peptides ($p<0.05$). These results suggest that TBP could serve as a valuable source of DPP-IV inhibitors. As illustrated in the regression analysis in Figure 4a, the IC_{50} value of LHIVGPDK was 1.41 mg/mL (1.61 mM). The inhibitory effect of LHIVGPDK was superior to those reported for LLPSY (IC_{50}: 2.48 mM) and NAPALVY (IC_{50}: 7.63 mM) from *Allium tuberosum* (Thi Phuong Nong et al., 2024); LTWR (IC_{50}: 1.79 mM) from *Musculus senhousei* (L. Zhou et al., 2024); IGL(IC_{50}: 2.22 mM) and GGGW (IC_{50}: 2.73 mM) from chicken blood (Carrera-Alvarado et al., 2022); FGPGP from discarded cowhide collagen (IC_{50}: 3.31 mM; He et al., 2023); and ADF, MIR, and FGR from egg (IC_{50}: 16.83, 4.86, and 46.22 mM,

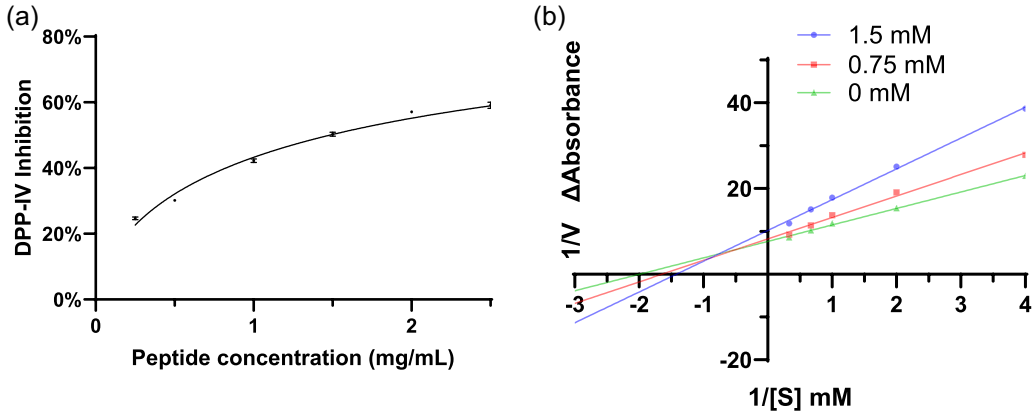

FIGURE 4 (a) Regression curve of the dipeptidyl peptidase-IV (DPP-IV) inhibition rate of Leu-His-Ile-Val-Gly-Pro-Asp-Lys (LHIVGPDK); (b) Lineweaver–Burk double reciprocal of LHIVGPDK.

respectively; Zhao et al., 2020). Staple foods have also been found to contain DPP-IV inhibitory peptides, such as IQAEGGLT from quinoa (250 μM, 17.05% inhibition; Chen et al., 2024), which is comparable to LHIVGPDK. However, LQAFEPLR from oats (IC_{50}: 103.5 μM) exhibited stronger inhibitory activity than LHIVGPDK (Wang et al., 2015). Additionally, peptides derived from animal-based proteins have demonstrated relatively strong DPP-IV inhibition, with IC_{50} values ranging from 5.61 (IPV from boarfish protein) to 259.20 μM (KPAGN from hydrolyzed tilapia skin; Harnedy-Rothwell et al., 2020; He et al., 2023; Li-Chan et al., 2012; Nongonierma & FitzGerald, 2013).

Although the newly discovered tartary buckwheat peptides showed better DPP-IV inhibitory activity compared to some previously reported peptides, they still exhibited lower activity than other reported peptides, likely due to tartary buckwheat's low digestibility, which resulted in larger peptide fragments. In general, peptides with fewer amino acids, especially di- and tri-peptides, tend to exhibit more potent DPP-IV inhibitory activity (Farias et al., 2022; Nongonierma & FitzGerald, 2019; Zheng et al., 2024). Furthermore, unlike some fractionation methods that show significant differences in DPP-IV inhibition among fractions (Gu et al., 2021), the TBP hydrolysate fractions obtained from ultrafiltration and RP-HPLC in this study also exhibited relatively high inhibitory effects. This suggests that the peptide mixture from TBP hydrolysate may synergistically inhibit DPP-IV at multiple sites, rather than relying on single peptide. In the future, employing other separation methods based on different properties, such as DEAE ion exchange chromatography, may help isolate more effective core DPP-IV inhibitory peptides from tartary buckwheat hydrolysates. Additionally, the IC_{50} values are influenced by assay conditions such as substrate and enzyme concentrations, making direct comparisons between different systems challenging. For example, varying concentrations of Gly-Pro-pNA substrate (25–500 μM) used in studies can significantly affect IC_{50} results (He et al., 2023; Mu et al., 2024; Wang et al., 2015; Y. Zhang et al., 2016).

Tartary buckwheat has been shown to benefit insulin resistance in diabetes mellitus (Qiu et al., 2016). This study represents the first report on the DPP-IV inhibitory properties of tartary buckwheat hydrolysates. This study emphasizes tartary buckwheat's potential as a DPP-IV inhibitor, contributing to its anti-diabetic properties. Beyond its recognized benefits from flavonoids and dietary fiber, this reinforces its role as a beneficial staple food for managing diabetes. Especially, by simulating the gastrointestinal digestion of TBP, this study provides valuable scientific evidence for understanding the generation of DPP-IV inhibitory effects from TBP during its digestion.

3.4 | Molecular mechanisms of DPP-IV inhibitory peptide LHIVGPDK

Figure 4b shows the inhibition mode of LHIVGPDK using Lineweaver-Burk double reciprocal plots. The linear regression curves for different concentrations of LHIVGPDK intersect in the second quadrant. As the concentration of active peptides increased, the *y*-axis intercept rose, while the *x*-axis intercept decreased. This trend indicates that with increasing peptide concentration, V_{max} gradually decreases, and K_m gradually increases, demonstrating a mixed inhibition type that includes both competitive and non-competitive inhibition (Y. Zhang et al., 2016; S. Zhang et al., 2023). These findings suggest that LHIVGPDK inhibits DPP-IV by binding to both the catalytic active center and non-active sites on the enzyme (S. Zhang et al., 2023).

In addition, molecular docking—a computational technique that simulates the interactions between ligands and target proteins—helps identify the binding sites and modes

TABLE 2 Modes of action between Leu-His-Ile-Val-Gly-Pro-Asp-Lys (LHIVGPDK) and dipeptidyl peptidase-IV (DPP-IV).

Interaction	Combination point
Hydrogen bonds	Glu 408(4.63 Å、3.92 Å、3.22 Å、5.13 Å)、Phe 461(3.85 Å)、Lys 463(5.74 Å)
Carbon-hydrogen bonds	Ser 460(4.44 Å)、Ser 158(5.62 Å)、Trp 157(6.60 Å)
Hydrophobic interactions	Trp 62(4.99 Å)、Lys 463(3.52 Å)
Electrostatic interaction	His 363(6.81 Å)
Van der Waals forces	Glu 361、Ala 409、Val 459、Leu 60、Ile 107、Arg 61、Ser 106、Thr 156、Pro 159、Ile 63、Pro 109

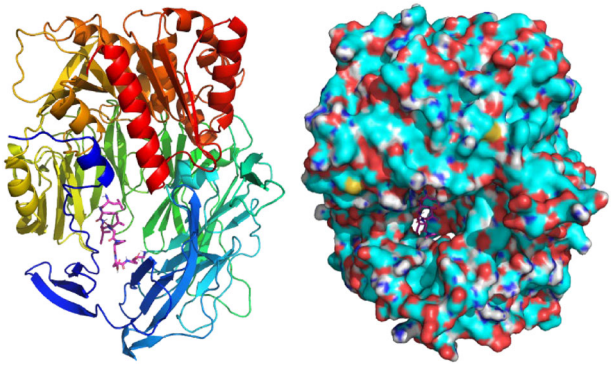

FIGURE 5 Molecular docking diagram of the interaction between Leu-His-Ile-Val-Gly-Pro-Asp-Lys (LHIVGPDK) and dipeptidyl peptidase-IV (DPP-IV).

of action of active peptides with DPP-IV. This approach further clarifies the relationship between the structure and efficacy of active peptides (Xu et al., 2019). Binding energy is a critical parameter for evaluating the stability of the system. Figure 5 shows the optimal docking conformations of LHIVGPDK with DPP-IV. In these conformations, the binding energy of LHIVGPDK was −2.77 kcal/mol. The negative binding energy values indicate that the LHIVGPDK can form stable complexes with DPP-IV. The binding primarily involves hydrogen bonds, hydrophobic interactions, electrostatic interactions, and van der Waals forces, with hydrogen bonds playing a key role in the interaction process. As shown in Table 2, LHIVGPDK interacts with the amino acid residues of the DPP-IV via six hydrogen bonds, three carbon–hydrogen bonds, two hydrophobic interactions, one electrostatic interaction, and 11 van der Waals forces. Except for Pro 159 and Trp 157, the amino acid residues involved in the binding have not been previously reported. Combined with the mixed competitive and non-competitive inhibition types shown in Figure 4b, this suggests that the active pocket of the DPP-IV enzyme may have new active sites, and some unreported amino acid residues outside the active center may also play a role. Therefore, while binding energy can partly explain the interaction between peptides and the enzyme, it is essential to further evaluate the inhibitory effect by comprehensively considering the docking sites and the magnitude of binding forces. Future studies should explore the in vivo efficacy of these peptides and their stability during food processing and digestion.

4 | CONCLUSION

The DPP-IV inhibitory peptides derived from TBP were identified and characterized. Among the five hydrolysis methods, SGID yielded the most active DPP-IV inhibitory hydrolysates, with the highest degree of hydrolysis and inhibition activity. Further enrichment was achieved using ultrafiltration (<3 kDa) and RP-HPLC resulting in F1, which contained 10 new peptides, including LHIVGPDK, which showed DPP-IV inhibition with an IC_{50} of 1.61 mM. The LHIVGPDK exhibits competitive and non-competitive inhibition mechanisms and binds both the active site and other residues of DPP-IV by molecular docking. This study underscores TBP's potential to inhibit DPP-IV, contributing to tartary buckwheat's anti-diabetic properties beyond its known benefits from other bioactive components. This reinforces tartary buckwheat's role as a beneficial food for managing diabetes.

AUTHOR CONTRIBUTIONS

Weijing Wu: Conceptualization; funding acquisition; investigation; writing—original draft; writing—review and editing. **Libo Wang**: Investigation; funding acquisition. **Ju Qiu**: Investigation; funding acquisition. **Ziyuan Zhuang**: Investigation. **Fei Qin**: Investigation. **Qianglai Tan**: Investigation; funding acquisition. **Yanling Wang**: Investigation. **Lanlan Wu**: Investigation; writing—review and editing; funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ORCID

Lanlan Wu https://orcid.org/0000-0002-1658-6777

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SUPPORTING INFORMATION

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