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Dipeptidyl Peptidase 4 Inhibitory Activity of Protein Hydrolysate from Earthworms (*Perionyx excavatus*)

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ABSTRACT

Diabetes is a complex metabolic syndrome, of which type 2 diabetes (T2DM), primarily caused by impaired insulin response, accounts for 95% of diabetes cases worldwide as of 2021. One treatment strategy to control T2DM includes inhibiting dipeptidyl peptidase-4 (DPP4), an enzyme that breaks down incretin hormones and impairs glucose regulation. This study explores the potential of hydrolysed proteins from earthworms (*Perionyx excavatus)* as natural DPP4 inhibitors, focusing on optimizing hydrolysis conditions, including enzyme type hydrolysis, earthworms:phosphate buffer (w/v), temperature (°C), pH, enzyme:substrate (E:S) ratio (U/g protein), and time hydrolysis (hour). Among the tested enzymes, Alcalase produced the most effective hydrolysate. The optimal conditions for hydrolysis were determined at an E:S ratio of 1:6 (w/v), a temperature of 55°C, pH 7, an enzyme: substrate ratio of 600 U/g protein, and a hydrolysis time of 4 hours. Under these conditions, the resulting hydrolysate exhibited vigorous DPP4 inhibitory activity (DPP4IA), with an IC_{50} value of 777.18 µg/mL. Further ultrafiltration fractionation revealed that the <1 kDa fraction had the highest inhibitory activity, with IC_{50} values of 395.03 and 323.07 µg/mL before and after digestion, respectively. Notably, the hydrolysate demonstrated stability across a broad pH range (1–11) and remained bioactive even after exposure to 100°C for up to 180 minutes. Interestingly, enzymatic hydrolysis and simulated digestion appeared to enhance bioactivity, likely due to the generation of smaller bioactive peptides. These findings suggest that peptides derived from *Perionyx excavatus* could be promising natural DPP4 inhibitors with potential applications in functional foods or pharmaceutical formulations for blood glucose management.

Keywords: Type 2 diabetes (T2DM), Dipeptidyl peptidase-4 (DPP4), *Perionyx excavatus*, protein hydrolysate, peptide fraction, hypoglycaemic activity.

INTRODUCTION

Diabetes is a common metabolic disease that is becoming increasingly prevalent among younger people, with the number of cases expected to reach 693 million by 2045 (Cho et al., 2018). Type 2 diabetes mellitus (T2DM) accounts for approximately 90–95% of all cases, mainly due to reduced insulin production or impaired insulin sensitivity (Aryan et al., 2018a). One key regulator of blood glucose is glucagon-like peptide-1 (GLP-1), which is released from enteroendocrine cells during digestion. GLP-1 enhances insulin secretion, suppresses glucagon release, and plays a critical role in glucose homeostasis. However, GLP-1, an enzyme that is present in large amounts in plasma and widely distributed in many organs, is rapidly degraded by dipeptidyl peptidase-4 (DPP4). Overactivity of DPP4 is associated with obesity and type 2 diabetes and may be a therapeutic target. Therefore, developing DPP4 inhibitors has become essential for controlling type 2 diabetes. Consequently, developing DPP4 inhibitors has become vital for managing type 2 diabetes (Müller et al., 2019).

Recently, the potential of bioactive peptides as natural DPP4 inhibitors has attracted much attention. The biological properties obtained from using enzymatic hydrolysis of peptides include antibacterial, anti-diabetic, and antihypertensive effects. Hydrolysis enhances peptides' bioactivity, digestibility, and bioavailability, making them more effective for clinical physiological applications (Lacroix & Li-Chan, 2016). In T2DM management, bioactive peptides have been recognized for their ability to inhibit metabolic enzymes such as dipeptidyl peptidase 4 (DPP4), a key target for glycaemic control (Luo et al., 2022). Various dietary protein sources, including dairy, eggs, fish, and plant-based options such as cowpea bean, amaranth, and quinoa, have been investigated for their potential to yield DPP4-inhibitory peptides (Lacroix & Li-Chan, 2016). These peptides control blood glucose levels by stimulating incretin hormones to enhance insulin release and regulate carbohydrate metabolism (McLaughlin et al., 2021). In addition to blood glucose control, bioactive peptides also play an essential role in reducing chronic inflammation associated with T2DM (Aryan et al., 2018b). By modulating cytokine pathways and lipid metabolism, these peptides contribute to metabolic balance and overall health improvement (Kamal et al., 2018).

Among alternative protein sources, *Perionyx excavatus*, an earthworm from the family Megascolecidae, has attracted attention due to its fast growth rate and strong regenerative ability. This species is crucial in organic matter decomposition and soil improvement (Byambas et al., 2019). Additionally, *P. excavatus* has long been used in traditional medicine for fever reduction, calming effects, blood circulation stimulation, and hypertension management (Girigoswami et al., 2025). Nutritionally, *P. excavatus* is particularly rich in protein, accounting for approximately 60–70% of its dry weight (Ngoc et al., 2016). Protein hydrolysis of this species generates bioactive peptides and enzymes with various potential biological activities, particularly in blood sugar regulation (Power et al., 2014). Previous studies have demonstrated that protein hydrolysate derived from *P. excavatus* can inhibit Angiotensin-I-Converting Enzyme (ACE), a key enzyme for hypertension (Bui et al., 2025). Besides, protein hydrolysate from *P. excavatus* can inhibit α-amylase and α-glucosidase, slowing carbohydrate breakdown and helping regulate postprandial blood glucose levels (Bui et al., 2023). However, research on the potential of *P. excavatus* peptides in inhibiting DPP4 has not been conducted. While DPP4 inhibitory peptides have been widely studied in dairy, fish, and plant-based proteins, *P. excavatus* remains an unexplored yet promising source (Liu et al., 2019).

Most previous studies have shown that hydrolysed proteins generally exhibit higher DPP-IV inhibitory activity compared to intact proteins. This can be explained by the structural characteristics of the DPP-IV enzyme, which has a narrow active site and preferentially binds short peptides, especially those containing proline or alanine at the N-terminus. Meanwhile, intact proteins have large molecular sizes and complex secondary and tertiary structures, limiting the enzyme's ability to access the catalytic centre. Additionally, peptide sequences with DPP-IV inhibitory potential are often "hidden" within the protein structure and cannot exert their activity unless released by hydrolysis (Miguéns-Gómez et al., 2021, Pérez-Gálvez et al., 2024a). Many studies have also shown that enzymes such as Alcalase, Flavorzyme, Neutrase, and Protamex can produce DPP-IV inhibitory peptides to varying degrees. Enzyme selection is crucial because the primary specificity and catalytic mechanism of each protease determine the protein cleavage pattern, thereby affecting the composition, size, and biological activity of the resulting peptide. Alcalase and Protamex are broad-spectrum endopeptidases, producing a wide variety of peptide fragments; Neutrase is a neutral metalloprotease with moderate specificity; while Flavourzyme possesses both endo- and exopeptidase activity, resulting in smaller peptides. These mechanistic differences lead to variations in peptide size distribution, thereby directly affecting DPP-IV inhibitory activity. Selecting the appropriate protease is key to efficiently releasing biologically active peptide chains from earthworm proteins (Wang et al., 2024a, Vasudevan Ramakrishnan et al., 2025). Therefore, screening different proteases is necessary to identify the optimal enzyme capable of producing DPP-IV inhibitory peptides from *P. excavatus.*

This study aims to evaluate the DPP4 inhibitory activity of peptides derived from the enzymatic hydrolysis of *P. excavatus* proteins using commercial proteases. The hydrolysis process will be optimized by assessing factors such as enzyme type, earthworm-to-buffer ratio, temperature, pH, enzyme-to-substrate ratio, and hydrolysis duration. The hydrolysate with the highest inhibitory activity will be fractionated based on molecular weight (kDa) to determine the most bioactive peptide fraction. Additionally, its stability under simulated gastrointestinal digestion, pH variations, and temperature fluctuations will be evaluated to assess its potential for practical applications.

MATERIALS AND METHODS

Include a clear description of materials, equipment, and methods in sufficient detail to allow repetition of the work elsewhere. Also, describe all safety considerations, including any procedures requiring special precautions, in sufficient detail so that those repeating the experiments can take appropriate safety measures. Published procedures should be cited, but not described, except for substantial modifications. Ethical clearance must be obtained for any study involving animal or human subjects.

Earthworms (*Perionyx excavatus*) purchased from the Biotechnology Center of Ho Chi Minh City were crushed and transported on ice to the Biochemistry laboratory of the Ho Chi Minh City University of Technology within 3 hours, individually wrapped in polyethylene bags, labelled, and refrigerated at -20°C until required. Earthworms are stored at -20°C and pureed. The earthworm sample contained 69.92 ± 0.23% crude protein, 6.97 ± 0.10% crude lipid, 12.78 ± 0.18% crude carbohydrate, and 10.25 ± 0.10% ash on a dry-weight basis, with an initial moisture content of 80.99 ± 0.53%.

Dipeptidyl Peptidase 4 (DPP4), Gly-Pro p-nitroanilide hydrochloride as a DPP4 substrate, and Diprotin A were purchased from Sigma Chemical Co. (USA). Protease enzymes (Alcalase® 2.5L, Neutrase® 0.8L, Protamex®, and Flavourzyme® 500MG) were purchased from Novozymes Co. (Bagsvaerd, Denmark). All other reagents used in this study were of the highest grade available commercially.

Instrumentation

This study used a UV-Vis 1800 spectrophotometer (Shimadzu, Japan), Mikro 200 centrifuge (Hettich, Germany), TC-NextSpin-P106 centrifuge (Taisite Lab Sciences Inc, USA), and Memmert incubator (Memmert, Germany). The 30, 10, 3, and 1 kDa ultrafiltration membranes were purchased from Pall Laboratory Co. (USA)

Preparation of Protein Hydrolysates

The preparation of hydrolysates was conducted using a method that had been modified previously (Bui et al., 2025). Sodium phosphate buffer (0.02 M Na_2HPO_4 - KH_2PO_4 buffer, pH 5.0 – 8.0) and Tris-HCl buffer (pH 8.0 – 9.0) were used to balance the pH. The appropriate ratio was added to a buffer solution (with a pH adequate for enzyme activity), and the combination was heated at 90 °C for 10 minutes to deactivate endogenous enzymes. After hydrolysis at a predetermined time, the enzymes were deactivated by heating the hydrolysates for 10 min at 90 °C. The supernatant was then collected by centrifuging the hydrolysates at 5000 rpm for 15 minutes. Other components, such as lipids and carbohydrates, are either insoluble in the aqueous buffer system or are removed by centrifugation after hydrolysis. The Lowry method determined that protein is soluble in hydrolysate. For use in the following experiments, a portion of the resulting supernatant was freeze-dried and stored at -20°C.

Effect of hydrolysis condition on the DPP4 inhibitory activity (DPP4IA) of protein hydrolysate from earthworms.

Alcalase (55°C, pH 8), Flavourzyme (50°C, pH 7), Neutrase (50°C, pH 7), and Protamex (55°C, pH 6.5) were selected for hydrolysis. The hydrolysis occurred in 4 hours with the enzyme: substrate ratio of 500 U/g protein and the earthworms: buffer ratio of 1:6 (w/v). Other hydrolysis conditions were examined with the hydrolytic enzyme that gave the highest DPP4IA.

The effect of factors on the DPP4 inhibitory capacity of proteolysis was examined using a single-factor test method, in which one factor was varied at different levels while the other factors remained constant. The factors in the study include earthworm: buffer ratio (w/v), temperature (°C), pH, E:S ratio (U/g protein), and hydrolysis time (h).

Fractionation of protein hydrolysate from earthworms.

From the highest DPP4IA protein hydrolysate, five peptide fractions were prepared and evaluated for their DPP4 inhibitory capacity: >30kDa, 10–30 kDa, 3–10 kDa, 1–3 kDa, and <1 kDa by using ultrafiltration centrifugal devices of 30 kDa, 10kDa, 3kDa, and 1kDa (Pall Laboratory, USA).

Determination of DPP4A inhibitory activity.

The method of evaluating DPP4IA was described in a previous study (Kamal et al., 2018). 25 µl of hydrolysate was mixed with 25 l of Gly – Pro – p – nitroaniline 12 mM and incubated at 37 °C for 10 min. Then add 50 µl DPP4 (0.02 U/mL) and incubate at 37°C for 30 min. Finally, add 100 µl of 1M sodium acetate buffer at pH 4 to terminate the reaction. The sample was measured at 405 nm. The DPP4 inhibitory activity was calculated based on the following formula:

$$\%\ \text{inhibition} = \frac{A-B}{A}.100\%$$

With A = OD control – OD control blank, and B = OD sample – OD sample blank.

OD control is the absorbance of the solution with the enzyme and substrate without the sample; OD control blank is the absorbance of the solution with the substrate without the enzyme and sample; OD sample is the absorbance of the solution with the enzyme, substrate, and sample; OD sample blank is the absorbance of the solution with the sample and substrate without the enzyme.

The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of DPP4 inhibitory activity. The logarithmic regression analysis calculated IC_{50} values. The sample performed similarly to the sample with expected concentrations of 0.0625 to 8 mg/mL. The positive control was that Diprotin A performed similarly to the sample with expected concentrations of 0.0005, 0.001, 0.002, 0.004, and 0.008 mg/mL.

Stability of DPP4 inhibitory peptides activity

During in vitro digestion, samples were collected at different time intervals to determine the residual DPP4 inhibitory activity. The activity was expressed as the percentage relative to the untreated control sample at 0 min, which was considered as 100% (Kang et al., 2020). The hydrolysate and fractions with a concentration of 5% (w/v) were adjusted to pH 2 with 6N HCl and incubated at 37 °C. The hydrolysis was performed by pepsin under conditions of an enzyme: substrate ratio of 4 % (w/w), 37 °C, and shaken at 230 rpm for 60 min. The pH was adjusted to pH 7.5 with 6N NaOH. The hydrolysis was performed by pancreatin at an E/S ratio of 4 % (w/w) at 37 °C and shaken at 230 rpm for 2 h. The mixture was heated at 90 °C for 10 minutes to inactivate the digestive enzymes. During *in vitro* digestion, samples were collected to determine the residual DPP4 inhibitory activity, which was expressed as the activity (%) relative to that without any treatment (control, 100%) at 0 min.

For thermal stability of DPP4 inhibitory peptide activity, 5 mL of peptide solutions with a concentration of 20 mg/mL were heated at 100 °C for 15, 30, 45, 60, 90, 120, 150, and 180 min, respectively. After that, the peptide solutions were brought to room temperature. The sample without heat treatment was used as the control. Then, the solutions were diluted to a final volume of 10 mL with deionized water. The DPP4 inhibitory activity stability after heat treatment was measured and expressed as the activity (%) relative to that without any treatment (control, 100%) (Sripokar et al., 2019).

For pH stability of DPP4 inhibitory peptide activity, 5 mL of peptide solutions with a concentration of 20 mg/mL were treated for 30 min at room temperature and pH 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0, after which the pH was adjusted to 7.0 by phosphate buffer 1.0 M. Then the solutions were diluted to the final volume of 10 mL with deionised water. The DPP4 inhibitory activity stability after pH treatment was measured and expressed as the activity (%) relative to that without any treatment (control, 100%) (Sripokar et al., 2019).

Statistical Analysis

All experiments were conducted in triplicate, and the obtained data were expressed as mean ± standard deviation and analysed using Excel software. Statistically significant differences were determined using SPSS software (IBM SPSS Statistics 20).

RESULTS AND DISCUSSION

Effect of condition hydrolysis on the DPP4IA of protein hydrolysate from earthworms

Effect of hydrolysis enzyme type on the DPP4IA of protein hydrolysate from earthworm

When hydrolysing earthworm protein with 4 enzymes, Alcalase, Flavourzyme, Neutrase, and Protamex, it showed the highest DPP4 inhibitory activity when using Alcalase for hydrolysis (Figure 1A). Previous studies have also reported that Alcalase-generated protein hydrolysates exhibited stronger DPP4 inhibitory activity than hydrolysates produced using other proteases. Similar findings were observed in hydrolysates prepared from *A. diaperinus* protein isolate (Lacroix et al., 2019) and sardine protein (Sarteshnizi et al., 2021).

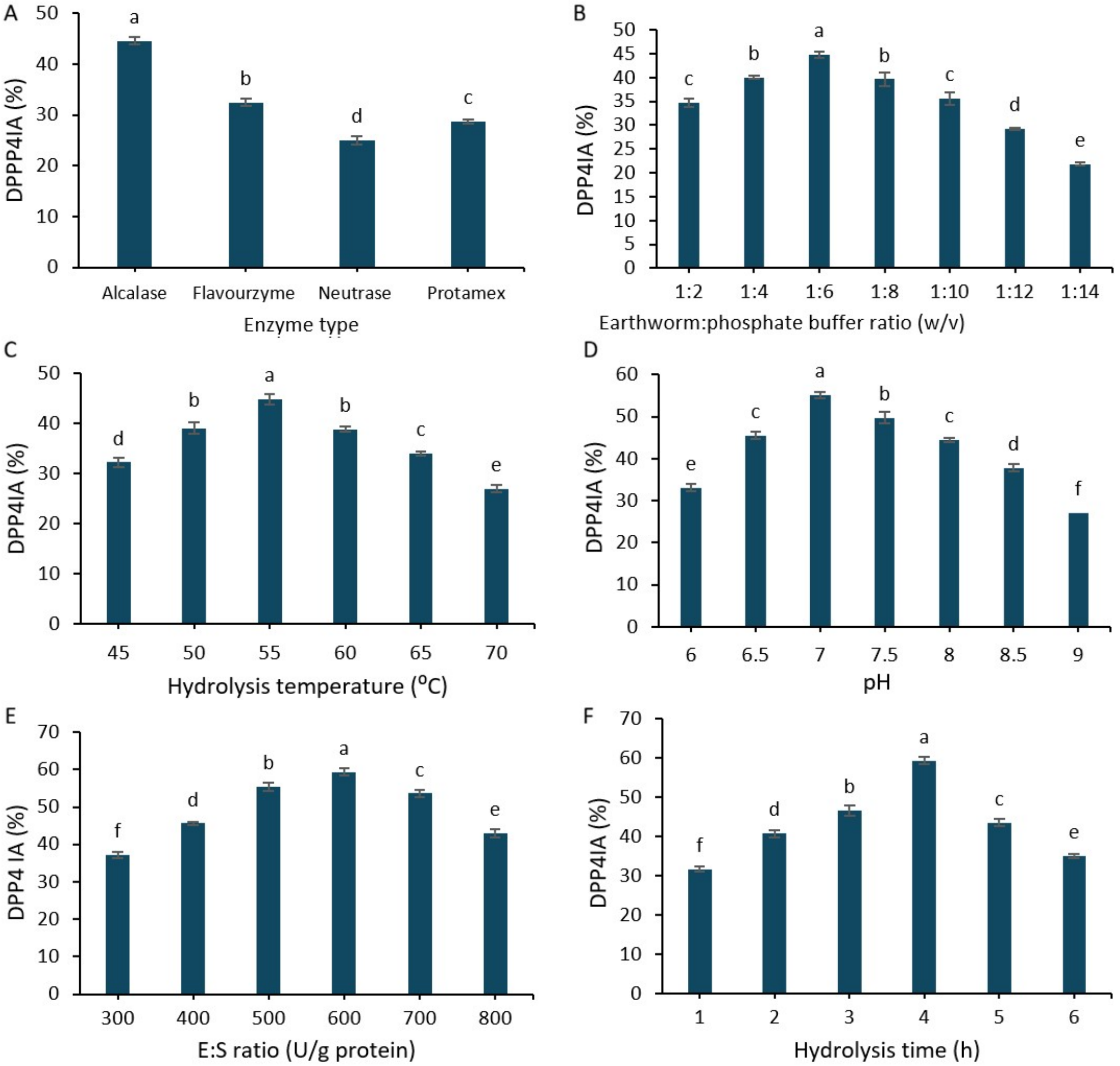

Figure 1. Effect of hydrolysis enzyme type (A), earthworm: buffer (w/v) ratio (B), hydrolysis temperature (°C) (C), pH (D), E:S ratio (U/g protein) (E), hydrolysis time (F) on the DPP4 inhibitory activity of protein hydrolysate from earthworms. Alcalase was selected for further optimization due to its highest DPP4 inhibitory activity among the test enzymes. The bars in each chart indicate the significant differences in the conditions according to their belonging letter ($p < 0.05$)

According to previous studies, the N-terminus of potent DPP-IV inhibitory peptides often contains long-chain amino acids (Leu, Ile) or aromatic amino acids with polar groups on the branched chain (Trp). Peptides with the ability to inhibit DPP4 are usually 2–6 amino acids and rich in hydrophobic and aromatic amino acids, such as Leu, Ile, Val, Phe, Tyr, Trp, and Pro (Wang et al., 2022). Earthworms contain many hydrophobic amino acids, such as Pro (accounting for 1.81% of dry weight), Leu (3.74% of dry weight), and Ile (2.74% of dry weight), so they can produce many peptides with strong binding ability to DPP-IV. In addition, the cleavage properties of Alcalase® 2.5 L (cleaving peptide chains at amino acids such as Gly, Leu, Ile at position P1 or amino acids Lys, Ile at position P2) (Fu et al., 2018) also produce peptides with high DPP-IV inhibitory activity. Thus, hydrolysis of earthworm protein using Alcalase® 2.5 L could produce peptides with DPP-IV inhibitory activity.

Effect of earthworm:phosphate buffer ratio on the DPP4IA of protein hydrolysate from earthworms

The results showed that the ratio between earthworm (*Perionyx excavatus*) and phosphate buffer solution significantly affected the DPP4 enzyme inhibitory ability of the protein hydrolysate. When the earthworm:buffer ratio was low (1:14, w/v), the protein hydrolysis efficiency reached the lowest level because the enzyme and substrate (earthworm protein) were too diluted, making it difficult to come into contact to perform the hydrolysis reaction. The results of the DPP4 inhibitory activity survey of the hydrolysate obtained at this ratio were also the lowest in the ratio range (21.88 ± 0.42%). On the contrary, when the earthworm:buffer ratio increased (the highest was 1:4, w/v), reflecting a low DPP4 enzyme inhibitory ability (34.67c ± 0.86%), the hydrolysis efficiency decreased because the reaction medium became too concentrated and highly viscous, limiting the contact between the enzyme and the substrate, leading to a decrease in the content of biopeptides capable of inhibiting DPP4 (Deng et al., 2018). When tested at a ratio of 1:6, w/v, the hydrolysate obtained showed the highest DPP4 inhibitory ability (about 44.82 ± 0.72%) (Figure 1B), indicating a balance between hydrolysis efficiency and bioactive peptide concentration. This result is consistent with the study by Deng et al. (2018), showing that the ratio of raw materials and buffer solution directly affects the bioavailability of peptides obtained from the hydrolysate (Deng et al., 2018). Determining the optimal ratio between earthworm and phosphate buffer solution is necessary to ensure the highest hydrolysis efficiency and DPP4 inhibitory activity.

Effect of temperature on the DPP4IA of protein hydrolysate from earthworms

From the results, it can be seen that temperature has a significant effect on the DPP4 enzyme inhibitory ability of earthworm protein hydrolysate (*Perionyx excavatus*). When the hydrolysis temperature increased from 45°C to 55°C, the DPP4 inhibitory activity tended to increase, and the inhibition rate reached a maximum at about 55°C (44.86 ± 1.03%) (Figure 1C). This can be explained by the fact that at the optimal temperature, the protease enzyme is most active, cleaving peptides with suitable structures to bind to the active centre of DPP4, thereby increasing the DPP4 enzyme inhibitory efficiency (López-Sánchez et al., 2016). However, as the temperature continued to increase above 55°C, the DPP4IA began to decrease. This may be due to the denaturation of the hydrolytic enzyme or the excessive degradation of bioactive peptides, which have lost the ability to interact with the enzyme. The above data are consistent with the research results of Bunsroem et al., 2022 on the impact of temperature on protein hydrolysis from whey protein isolate, showing that temperature control is an important factor in the process of creating peptides with high biological activity.

Thus, the optimal temperature for obtaining peptides from earthworm hydrolysate with the ability to effectively inhibit DPP4 is around 55°C. Determining appropriate temperature conditions helps improve hydrolysis efficiency and optimizes the biological activity of the obtained peptides, opening up potential applications in the development of functional foods to support the treatment of type 2 diabetes.

Effect of pH on the DPP4IA of protein hydrolysate from earthworms

The findings (Figure 1D) indicate that earthworm protein exhibits DPP4IA within the pH range of 6 to 9. Specifically, the DPP4IA increases from 32% at pH 6 to a peak of 58% at pH 7. However, from pH 7.5 to 9, the inhibitory activity gradually declines, reaching a minimum of 28%. This variation is statistically significant ($p < 0.05$), highlighting the direct impact of pH on the DPP4IA of protein hydrolysate from earthworms. The structural dependence of proteins in various pH conditions accounts for this difference. In addition, the kinetic nature of the enzyme indicates that the environmental pH affects the charge and substrate binding ability of the enzyme, which ultimately affects its stability, activity, and efficiency (Shu et al., 2016). Under optimal pH conditions, the hydrolysed protein maintains the appropriate conformation to achieve maximum DPP-4 inhibitory activity. Deviation from the optimal pH range may alter the protein structure and weaken its binding ability to DPP-4. Therefore, pH 7 was selected for the subsequent experiments, which is consistent with previous research findings (Divya et al., 2020).

Effect of E:S ratio on the DPP4IA of protein hydrolysate from earthworms

The study showed that DPP4IA was positively correlated with the E:S ratio in the range of 300 to 600 U/g protein. As illustrated in Figure 1e, DPP4IA peaked at approximately 60% when the E:S ratio reached 600 U/g protein. This increase can be attributed to the higher enzyme concentration, which enhances interactions with the substrate surface, breaking peptide bonds that are sensitive to enzymatic action. Therefore, the hydrolysis process is expedited, generating more DPP4IA peptides. However, when the E:S ratio went over 600 U/g protein, the DPP4IA of earthworm protein hydrolysate gradually declined. This may be because a continuous increase in the E:S ratio after the initial hydrolysis stage causes the reaction to proceed too rapidly, leading to the non-selective degradation of biologically active peptides. This finding indicates that the DPP4IA of earthworm protein hydrolysate is significantly influenced by the E:S ratio, consistent with previous studies (Li et al., 2023). Therefore, an E:S ratio of 600 U/g protein was identified as the optimal condition for subsequent experiments.

Effect of hydrolysis time on the DPP4IA of protein hydrolysate from earthworms

By investigating different hydrolysis durations under the available conditions, it was determined that 4-hour hydrolysis yielded the highest DPP4 inhibitory activity. As shown in The DPP4IA of the protein hydrolysate gradually increased with hydrolysis time, peaking at 4 hours (59.42%) (Figure 1F). This trend is similar to the study on whey protein hydrolysate from mare's milk (Song et al., 2017) and the survey on *Amaranthus hypochondriacus* (López-Sánchez et al., 2016) in which the DPP4IA reached its optimum level after 4 hours of hydrolysis. The increase in activity in the early stage may be due to the enzyme breaking down the primary, secondary, and quaternary structures of earthworm proteins, releasing peptides with potent biological activity. However, when the hydrolysis time was extended from 5 to 6 h, the DPP4IA gradually decreased, possibly due to the further cleavage process causing the active peptides to be broken down, leading to the loss of the structure necessary for effective interaction with the DPP4. In addition, this process can produce hydrolysates with low molecular weight but less inhibitory ability to the enzyme (Sangsawad et al., 2022). Therefore, a hydrolysis time of 4 h was considered optimal to obtain protein hydrolysates with the highest DPP4 inhibitory activity.

The IC_{50} value of DPP4IA of protein hydrolysate from earthworms

Under the hydrolysis conditions investigated, the IC_{50} value of the protein hydrolysate from earthworms with the highest DPP4IA was 777.18 (µg/mL). This value is lower than the hydrolysate of Bambara bean protein by Alcalase (IC_{50} value of 1730 µg/mL) (Mune et al., 2018), *P. palmata* protein by Alcalase (940 µg/mL) (McLaughlin et al., 2021), but higher than the hydrolysate of protein oat (IC_{50} value is 413 µg/mL) (Fuentes et al., 2021). Compared to Diprotin A, a standard DPP4 inhibitor, Diprotin A exhibited significantly more vigorous DPP4 inhibitory activity with an IC_{50} value of 1.5 (µg/mL), indicating that although the protein hydrolysate from earthworms showed moderate activity, it was less effective than Diprotin A.

DPP4IA of Peptide Fractions

The DPP4IA was highest in the <1 kDa fraction, followed by the 1 - 3 kDa, 3 - 10 kDa, 10 - 30 kDa, and > 30 kDa fractions (Figure 2). The results of this study determined that the low molecular weight peptide fraction had higher DPP4IA than the high molecular weight peptide fraction, similar to previous studies. Hydrolysed Atlantic Salmon Skin Gelatine was filtered through two membranes of 1 kDa and 2.5 kDa; the fraction < 1 kDa was also the fraction with the highest DPP4 inhibitory activity, with an IC_{50} value of 1350 µg/mL (Li-Chan et al., 2012). Filtering fractions of hydrolysates from Silkworm pupae (*Bombyx mori*) proteins by membrane filtration also obtained the highest DPP4 inhibitory activity in the <1 kDa fraction (Luo et al., 2022). Among the membrane filtration fractions, the <1 kDa fraction also had the highest DPP4 inhibitory activity, as in porcine skin gelatine hydrolysates, sodium caseinate hydrolysate (Hsieh et al., 2016), Portuguese oyster (*C. angulata*) proteins (Gomez et al., 2019).

Potent DPP4 inhibitory peptides typically contain 2–6 amino acids. Due to their small size, these amino acids easily access the active site of DPP4 (Wang et al., 2024b). In addition, the amino acid sequence of the peptide also affects the ability to inhibit DPP4 because it determines the formation of bonds between the peptide's amino acids and the amino acids in the active site of DPP4.

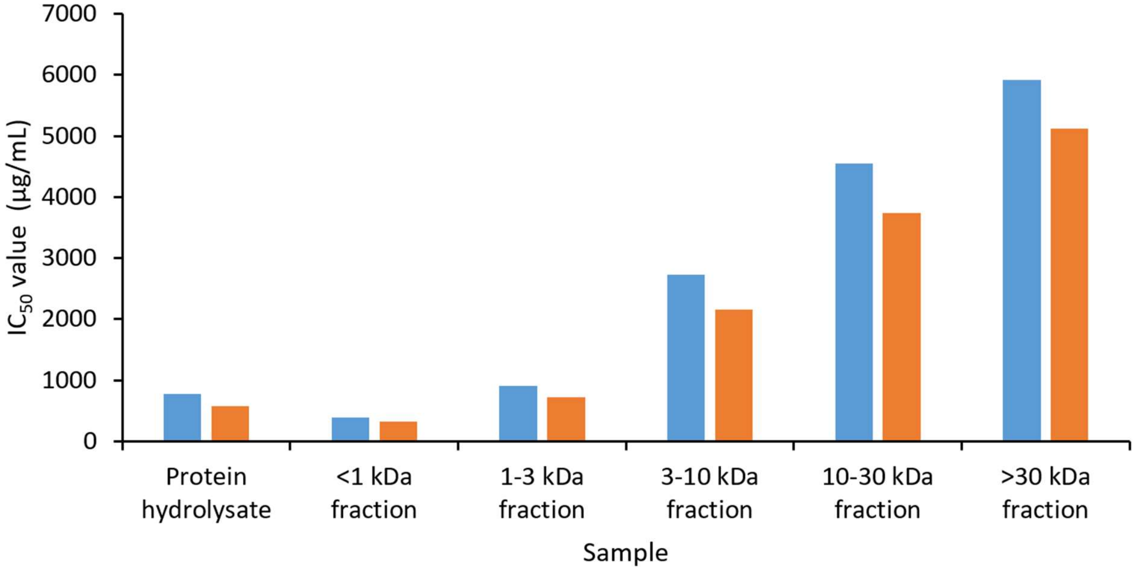

Figure 2. DPP4IA is demonstrated with the half inhibitory concentration, which is IC50 (µg/mL), of the earthworm protein hydrolysate and their five peptide fractions before and after the simulated digestion. All experiments were performed in triplicate (n = 3).

These peptides often contain hydrophobic and aromatic amino acids such as Leu, Ile, Val, Phe, Tyr, Tryp, and Pro. Having Pro at the second position (P2) or near the N-terminal helps the peptide bind well to the active pocket of DPP4 (Wan et al., 2023). Earthworm contains a lot of Pro, Leu, hydrophobic, and basic amino acids such as Met, Ile, and His...so it can create many peptides with a strong binding ability with DPP4 (Istiqomah et al., 2009). From the above data, it can be seen that small-volume peptide fractions from earthworm protein hydrolysate have strong DPP4IA.

Stability of DPP4IA of the hydrolysates and their peptide fractions

In vitro digestion stability of DPP4IA of the hydrolysates and their peptide fractions

When investigating the ability to inhibit DPP4 in different fractions, the lower IC_{50} value represents higher inhibitory activity. After *in vitro* digestion, the DPP4IA of the protein hydrolysates and fractions increased. The most substantial increase was in the protein hydrolysate and the 3-10 kDa fraction, as well as the 1-3 kDa fraction (Figure 2). Low molecular weight peptides retain DPP4 inhibitory activity even after digestion, showing high stability (Sorokina et al., 2023, 2024). The research findings are consistent with other investigations, including identifying peptides present in melon seeds (Hung et al., 2024), eggs of carp (*Cyprinus carpio*) (Zhang et al., 2020). Peptides with low molecular weight and efficient binding to the enzyme active site can change the conformation and block the substrate binding sites (Wang et al., 2024b). The DPP4 inhibitory activity of the peptide fractions is closely related to their structure, including peptide length, amino acid composition, and sequence. Short-chain peptides with specific amino acid residues, such as Pro and Ala, at the N-terminal position are more likely to exhibit vigorous DPP4 inhibitory activity (Xu et al., 2024; Zhang et al., 2024).

The increase in DPP4IA may be due to some peptides being resistant to cleavage by digestive enzymes. In contrast, some peptides may be cleaved and generate new peptides with higher DPP4 inhibitory potency than the original. Some peptides may resist peptidases in the gastrointestinal tract, especially short-chain peptides and peptides containing Pro (proline) (Zhang et al., 2022). Simulated digestion can increase DPP4IA, as the digestion process may release additional bioactive peptides with higher inhibitory potential (Pérez-Gálvez et al., 2024b).

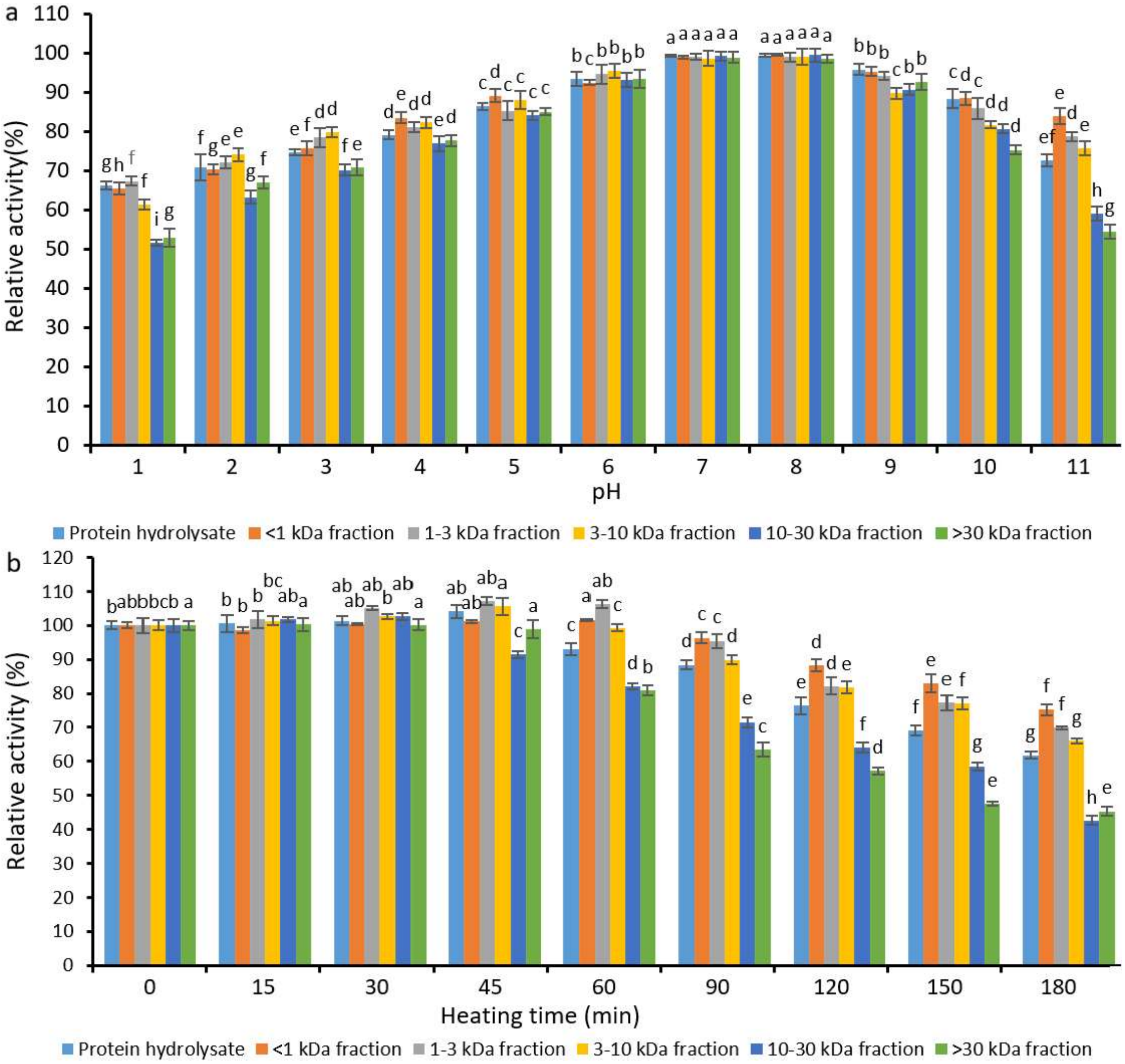

Figure 3. (a) pH stability and (b) thermal stability of DPP4IA of the earthworm protein hydrolysate and their five peptide fractions. The bars in similar color with different letters indicate the significant differences ($p < 0.05$)

Under optimal operating conditions, Pepsin efficiently cleaves peptide bonds, especially those adjacent to aromatic amino acids such as Leu, Phe, and Tyr (Vreeke et al., 2023). Pancreatin consists of various enzymes, including trypsin and chymotrypsin, which target specific amino acids for cleavage, such as Lys, Arg, Leu, and aromatic residues (Blanco & Blanco, 2017). Among them, leucine, lysine, and arginine are present in the protein composition of earthworms. From these observations, it can be shown that *in vitro* digestion can increase the DPP4IA of protein hydrolysates and their fractions from earthworms. Thus, earthworm protein hydrolysates and their fractions have potential for the development of oral anti-diabetic products.

pH stability of DPP4IA of the hydrolysates and their peptide fractions

At low pH values (<4) and high pH values (>9), the activity of protease enzymes is significantly possibly due to changes in peptide charge, especially at the N-terminus and C-terminus in highly acidic and alkaline conditions

(Ketnawa et al., 2017), changing electrostatic interaction forces and hydrogen bonds, changing the structure and solubility of the peptide, leading to reduced biological activity (Klomklao & Benjakul, 2018). At low pH (<4), high H^+ ion concentrations can break hydrogen bonds, causing the loss of the original structure of the enzyme, reducing enzyme activity. At high pH (>9), high OH^- concentrations change the ionization state of crucial amino acid residues, disrupting the spatial structure and reducing enzyme activity (Klomklao & Benjakul, 2018). Experimental results showed that at pH values that were too low or too high, the enzyme could be unstable, reducing its ability to bind to the substrate, thereby reducing enzyme activity. At pH 7 and 8, the hydrolysates and their fractions showed the highest activity stability, with the highest activity expression being approximately 100%. Compared to other pH values, pH 7 and 8 had the best activity stability in DPP4 inhibition. Proteins possess a low net electrostatic repulsive energy, making them stable mainly in the neutral pH range. This stability helps to minimize swelling and unfolding of protein molecules, thereby maintaining their biological activity (Klomklao & Benjakul, 2018).

The activity between fragments (<1 kDa, 1-3 kDa) has small differences; peptides of different sizes are similarly affected by pH, indicating that the main effect is the change in enzyme structure. The fraction (3-10 kDa) showed the highest activity (around 99% at pH 7-8) (Figure 3a), indicating that the enzyme was highly active in this size range. The more significant fraction (10-30 kDa) was less denatured than the small peptides, but was still affected by the strongly acidic environment, which changed the enzyme structure. The fraction (>30kDa) exhibited the lowest activity among all the fractions (52.99% at pH 1 and 75.41% at pH 10). Short peptide DPP-IV inhibitors are less affected by pH because of their simple structure, few ionizable groups, no dependence on higher order structure, and easy access to the enzyme active site, despite slight changes in pH (Guan et al., 2023).

pH affects long peptides more strongly because many ionizable groups (on the R side chains of amino acids: Lys, Glu, Asp, His, Arg, etc.) should change pH, changing the overall charge, causing loss of ionic interactions and higher-order structure. At the same time, long-chain peptides are more susceptible to hydrolysis or denaturation under extreme pH conditions, reducing the ability to interact with target enzymes (Masson & Lushchekina, 2022).

Thermal stability of DPP4IA of the hydrolysates and their peptide fractions

According to experimental results (Figure 3b), the activity stability of the protein hydrolysate and fractions seems to decline progressively with heating time. Some fractions displayed a minor increase during the first 45 minutes, followed by a dramatic decline, possibly due to the rearrangement of peptide structure or the impact of heat on the remaining enzymes in the sample. However, the activity started to decrease significantly after 60 minutes, suggesting that long-term temperature impacts affected the fractions' stability. The DPP4 inhibitory activity of every sample in this experiment remained constant throughout the initial heat treatment. This may be due to specific amino acids found in peptides that improve their thermal stability. For instance, Ile contributes to packing the inner core of the protein firmly, reducing its blank space. Therefore, the polypeptide chain's flexibility is reduced at high temperatures, decreasing its structural disorder and enhancing protein/peptide stability (Fennema et al., 2017). Val, Leu, and Phe were also found to play a similar role to Ile in protein stabilization (Nourmohammadi et al., 2017). In 45-90 min, the 1-3 kDa and 3-10 kDa fractions showed a slight increase in activity, as well as in the ACE inhibitory stability study, temperature affected hydrogen bonds, ionic bonds, or Van der Waals bonds, causing the peptide chain to straighten (Tamoliūnas & Galamba, 2020) and increase DPP4 inhibitory activity. However, the biological activity stability of these samples gradually decreased when the continuous heating time was extended to 180 min.

This result is similar to the previous study, which showed that with different heating times, the biological activity of peptides decreased after the threshold value (Wali et al., 2017). The reason may be protein denaturation, aggregation, and amino acid degradation. High temperature disrupts intermolecular and intramolecular interactions, exposing hydrophobic regions and causing protein aggregation (Klomklao & Benjakul, 2018). This change in activity is also a result of random remodelling of intramolecular disulfide bonds (Ninomiya et al., 2018).

Temperature is considered the most common factor affecting protein stability. High temperatures not only disrupt non-covalent interactions (hydrophobic interactions, electrostatic interactions, and hydrogen bonds) but also increase the denaturation of protein molecules, causing protein instability and loss of their biological activity (Fennema et al., 2017). Low molecular weight peptides are more stable at high temperatures because they are less linked than longer chain peptides. In contrast, large peptides are prone to aggregation due to hydrophobic interactions, reducing accessibility to the DPP4 enzyme. Similar findings were reported in studies on canola and silver carp protein hydrolysates, in which low molecular weight peptides exhibited more vigorous DPP4 inhibitory activity (Xu et al., 2019). When comparing the fractions, smaller peptides (<1 kDa, 1–3 kDa) were more heat-resistant than larger fractions. After 180 min, the <1 kDa fraction retained approximately 75% of its activity, while the >30 kDa fraction retained only approximately 45%. This indicates that the peptide from earthworms has relatively good thermal stability under the tested heating conditions and can withstand heat treatment conditions during processing or purification without significantly degrading its biological activity. The larger peptides are more susceptible to denaturation or hydrolysis upon prolonged heat exposure. Besides, the changes in the bioactivity of peptides after heat treatment may vary depending on the peptide type, size, and hydrophobic domain ratio (Kittiphattanabawon et al., 2012).

Although the DPP4 inhibitory activity of hydrolysed protein extract and peptide fragments from earthworms was moderate, the results are still significant. The spatial structure and distribution of the DPP4 enzyme may partially explain the moderate inhibitory activity. Therefore, the use of hydrolysed protein extract or peptide fragments from earthworms is still considered a potential agent for glycaemic control through DPP4 inhibition. In the context of the next-line multi-target glycaemic control approach, the DPP4 inhibitory activity of hydrolysed earthworm protein extract becomes even more valuable when simultaneously exhibiting the ability to inhibit α-amylase and α-glucosidase (Bui et al., 2023). Simultaneous action on multiple biological targets may contribute to enhancing its potential application in supporting glycaemic regulation. These results indicate that low molecular weight peptides have relatively stronger compared with higher molecular weight fractions DPP4 inhibitory activity and remain stable under prolonged thermal conditions. Therefore, ultrafiltration helps to enrich low molecular weight peptides and improve DPP4 inhibitory activity.

CONCLUSION

This study determined the optimal hydrolysis conditions for obtaining vermicompost protein hydrolysate with DPP4IA, in which Alcalase gave the highest yield. The optimal conditions included a substrate:buffer ratio of 1:6 (w/v), temperature of 55°C, pH 7, enzyme:substrate ratio of 600 U/g protein, and hydrolysis time of 4 h. The hydrolysate obtained under these conditions exhibited significant DPP4 inhibitory activity, especially in the <1 kDa fraction with an IC_{50} value of 395.03 μg/ml. Although this activity was lower than that of Diprotin A, it was significantly improved after *in vitro* digestion, increasing from 1.16 to 1.36 times. In addition, the hydrolysate and peptide fractions showed high stability, retaining more than 50% activity in a wide pH range (1-11) and 40% after being heated at 100°C for 180 min. These findings suggest the potential application of vermicompost protein hydrolysate as a source of biopeptides with DPP4 inhibitory ability, contributing to developing products supporting diabetes treatment. Further research should focus on identifying the major bioactive peptides and elucidating their mechanisms of action. In addition, in vivo studies using appropriate animal models are necessary to evaluate their hypoglycaemic efficacy and safety profile, thereby providing a scientific basis for their development as functional food ingredients supporting blood glucose regulation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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