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Earthworm (*Perionyx excavatus*) Protein Hydrolysate: Hypoglycemic Activity and Its Stability for the Hydrolysate and Its Peptide Fractions

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Abstract: This study aims at exploring earthworm protein hydrolysate and its peptide fractions as a potential hypoglycemic agent by inhibiting α-amylase and α-glucosidase. Firstly, the best hydrolysis conditions to gain the hydrolysates with the highest α-amylase inhibitory activity (α-AIA) and α-glucosidase inhibitory activity (α-GIA) were figured out using a one-factor test. Next, the stability of the hypoglycemic activity of the hydrolysates and their 5 peptide fractions recovered using ultrafiltration membranes were assessed by employing the tests of in vitro digestion, thermal, and pH treatment. The results showed that at the best hydrolysis conditions, the hydrolysates exhibited α-AIA of 91.30 ± 2.51% and α-GIA of 44.69 ± 0.47%. Specifically, the <1 kDa peptide fraction from the hydrolysate expressed a greater α-AIA than that of acarbose, with nearly the same α-GIA as that of voglibose. The α-AIA and α-GIA of the hydrolysates and their fractions were enhanced after the in vitro digestion treatment, whereas they remained over 40% after the pH treatment in the range of 1 to 11 or heat treatment at 100 °C for 180 min. These data provide the preliminary evidence to develop the earthworm protein hydrolysate and its peptide fractions in functional food or nutraceutical products with hypoglycemic activity.

Keywords: α-amylase inhibitory activity; α-glucosidase inhibitory activity; earthworm protein; antidiabetic activity; hypoglycemic activity; earthworm protein hydrolysate; peptide fraction

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1. Introduction

Earthworms are macro-invertebrates (*oligochaeta*), shouldering the responsibility to enhance soil fertility and productivity [1]. *Perionyx excavatus* is a popular species in Vietnam that has high protein content (55–70%, dry weight basis), thus it has been used as an alternative protein ingredient to feed common carp (*Cyprinus carpio* L.) [2], as well as a protein source for human in Asia, including India, Myanmar, China, Korea, and Vietnam for thousands of years [3]. The earthworm protein and its coelomic fluid were proven for their cytolytic, agglutinating, proteolytic, haemolytic, mitogenic, antipyretic, and tumorstatic activities [4]. Ding et al. [5] also documented that the earthworm was a potential source of pharmaceutical compounds expressing anti-hypertensive, anticoagulant, and anti-hyperlipidemic activities. Accordingly, it is interesting to utilize this high protein source and discover hidden bioactivities of this species.

The International Diabetes Federation (IDF) reported that in 2019, there were 463 million adults aged 20–79 years who had diabetes, and the number was predicted to reach over 700 million by 2045 [6]. Approximately 90% of diabetic patients had Type 2 diabetes, which is characterized by hyperglycemia (high blood glucose level) resulting from insulin-related disorders [7]. To control postprandial hyperglycemia and manage

diabetes mellitus, the delay of glucose release into the bloodstream by inhibition of starch-digestive enzymes (including α-amylase and α-glucosidase) is one of the most effective strategies [8,9]. Iminosugar-derivatives (such as miglitol) and sugar-derivatives (including acarbose and voglibose) have been used as synthetic antidiabetic drugs [7,10]. However, their side effects, such as gastrointestinal disorders, bloating, stomach pain, diarrhea, and flatulence, need to be considered [11].

Earthworm-derived products, including dried earthworms, earthworm powder, and extract, have been used as medicinal ingredients to treat allergies in China [12]. In addition, in various countries such as Taiwan, Japan, South Africa, Brazil, and the Philippines, earthworms have been consumed in their meals [5]. Moreover, earthworm protein powder (BOCOM XUETONG) and smoked earthworms have been marketed in the USA and Venezuela [5]. In addition, commercial Lumbrokinase, a serine protease from earthworms, is globally available for anti-allergy [13]. Moreover, earthworm meals were made and evaluated safely in terms of microbiology, heavy metals, pesticide, mycotoxins, and antibiotic residues [14,15]. Recently, food wastes (fruit and vegetable residues and kitchen waste) have been utilized to raise earthworms for sustainable protein source recovery and meet global protein needs [16,17].

Regarding the nutritional value of earthworm protein hydrolysate, Alcalase hydrolysate was reported to be rich in essential amino acids (14.7 g/kg of hydrolysate) and expressed high protein digestibility in vitro (73%) [18]. Moreover, high α-AIA and α-GIA of earthworm extract have been found by Mir et al. [19]. Furthermore, Abdelaziz et al. [20] discovered that the therapeutic potential of earthworm extract against diabetic complications mainly depends on its amino acid composition when performing an in vivo test on diabetic rats. In addition, the peptide's bioavailability was determined by its length, amino acid composition, charge, and hydrophobicity, and the peptide length might be the most important factor [21]. Patil et al. [22] found that for the small intestine, the absorbability of short-chain peptides (<6 amino acids) was more than 90% [9], which was about 45 times higher than that of acarbose [23]. Moreover, some animal-derived peptides were found to be hypoglycemic agents [11,24,25], due to their high affinity, specificity, bioactivity, and safety [26,27]. Our study is the very first to generate earthworm protein hydrolysate with hypoglycemic activity.

It was reported that smaller peptides may be more active than bigger peptides [28]. In addition, Félix-Medina et al. [29] and Li et al. [30] found that a better hypoglycemic activity of peptide fractions was observed as their molecular weights decreased. Moreover, Wang et al. [31] discovered that most peptides possessing antihyperglycemic activity have molecular weights below 1 kDa.

Félix-Medina et al. [29] published that α-AIA and α-GIA of the <3 kDa peptide fraction from maize protein hydrolysate were over 50% and 35%, respectively, after being treated at a temperature range from 30 to 90 °C or pH in the range of 2–8. In addition, the retention rate of α-GIA of hypoglycemic peptides in Moringa oleifera seed protein hydrolysates at pH = 2, 4, 6, and 8 was greater than 90%, however, it reduced to 60% at pH = 10 [31]. Meanwhile, their α-GIA could remain 100% at temperatures of 20, 40, and 60 °C, then quickly decreased to 18.73% at 100 °C [31]. On the other hand, the α-GIA of YPVEPF (a hypoglycemic peptide identified from whey hydrolysate) increased up to 4 times at pH = 2, and it reduced by approximately 9 times at pH = 10 [30]. Besides, this peptide was found to be stable at the temperature range of 20–80 °C, with its highest α-GIA retention rate of 94% at 60 °C [30].

Therefore, this study focused on investigating the α-AIA and α-GIA of the protein hydrolysate/peptide from earthworms. Firstly, the single factor test was employed to select the hydrolysis condition (enzyme type, earthworm: phosphate buffer ratio (w/v), temperature, pH, enzyme: substrate (E:S) ratio, and hydrolysis time) to obtain the hydrolysate that expresses the highest α-AIA and α-GIA. Subsequently, the hydrolysates were further fractionated using ultrafiltration membranes and 5 peptide fractions (>30 kDa, 10–30 kDa, 3–10 kDa, 1–3 kDa, and <1 kDa) were recovered and tested for their bioactivities. Then, the

hydrolysates and 5 peptide fractions were assessed for their bioactivity stability using the tests of in vitro digestion, thermal, and pH treatment.

2. Materials and Methods

2.1. Materials

Earthworms (*Perionyx excavatus*) were purchased from the Biotechnology Center in Ho Chi Minh City, then they were crushed and stored in polyethylene bags at −20 °C before testing. The chemical compositions of the earthworms were determined using the methods of AOAC (1990) [32]. The earthworms contained 80.99 ± 0.53% moisture, 69.92 ± 0.23% crude protein, 6.97 ± 0.10% crude lipid, 12.78 ± 0.18% carbohydrate, and 10.25 ± 0.10% ash (on a dry weight basis).

In this study, α-glucosidase (from *Saccharomyces cerevisiae*, 10 units/mg protein), p-Nitrophenyl α-D-glucopyranoside (pNPG), α-amylase (from the porcine pancreas, 5 units/mg solid), and acarbose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Enzyme preparations, including Alcalase® 2.5 L (55 °C, pH = 8), Neutrase® 0.8 L (50 °C, pH = 7), Protamex® (55 °C, pH = 6.5), and Flavourzyme® 500 MG (50 °C, pH = 7), were bought from Novozymes Co. (Bagsvaerd, Denmark) and AB enzymes (Darmstadt, Germany). All reagents were of analytical grade.

2.2. Methods

2.2.1. Preparation of Earthworm Protein Hydrolysates

Earthworm hydrolysates were prepared based on the method described in Vo et al. [33] with slight modifications. The ground earthworm was mixed with 0.02 M phosphate buffer with an appropriate ratio so that the pH reached the required value for hydrolysis, and the mixture was heated at 90 °C for 10 min to deactivate endogenous enzymes. Then, the pH was readjusted using 1 M HCl or 1 M NaOH solution, and the temperature was controlled to the hydrolysis temperature before adding enzyme preparation with the appropriate E:S ratio. At a set time, the enzyme was deactivated by heating the hydrolysate for 10 min at 90 °C. The supernatant was then collected by centrifuging at 5000 rpm for 15 min, and the obtained supernatant was lyophilized and stored at −20 °C. The soluble protein content of the hydrolysate was determined by the Lowry method [34].

2.2.2. Effect of Hydrolysis Condition on α-AIA and α-GIA of the Earthworm Protein Hydrolysate

The effect of hydrolysis parameters, including enzyme type, earthworm: phosphate buffer ratio (*w*/*v*), temperature (°C), pH, E:S ratio (U/g protein), and hydrolysis time (h), on α-AIA and α-GIA of the earthworm protein hydrolysates was investigated using the single factor test method. More specifically, one factor varied at different levels while the others remained constant, as presented in Table 1.

Table 1. Hydrolysis condition of each investigation.

Tested Parameter	X1 *	X2 *	X3 *	X4 *	X5 *	X6 *
Investigation 1	4 proteases **	1:6	Optimal **	Optimal **	500	4
Investigation 2	X ***	Tested range	Optimal **	Optimal **	500	4
Investigation 3	X ***	X ***	Tested range	Optimal **	500	4
Investigation 4	X ***	X ***	X ***	Tested range	500	4
Investigation 5	X ***	X ***	X ***	X ***	Tested range	4
Investigation 6	X ***	X ***	X ***	X ***	X ***	Tested range

* X1: enzyme type; X2: earthworm:phosphate buffer ratio (*w*/*v*); X3: temperature (°C); X4: pH; X5: E:S ratio (U/g protein); and X6: hydrolysis time (h). ** Value is shown in Table 2. *** The chosen level of the hydrolysis parameter after each investigation.

Table 2. Optimal pH and temperature of each enzyme preparation.

Enzyme Preparation	Optimal pH	Optimal Temperature
Alcalase® 2.5 L	8.0	55 °C
Neutrase® 0.8 L	7.0	50 °C
Protamex®	6.5	55 °C
Flavourzyme® 500 MG	7.0	50 °C

2.2.3. Determination of α-AIA and α-GIA of the Earthworm Protein Hydrolysates and Their 5 Peptide Fractions

The method described in Liu et al. [35] was employed with minor modifications to determine α-AIA of the protein hydrolysates. In brief, 0.20 mL of the sample (2 mg protein/mL) was incubated with 0.04 mL of α-amylase (5 U/mL) and 0.36 mL of sodium phosphate buffer (0.02 M, pH = 6.9) containing 6 mM NaCl at 37 °C for 20 min. Then, 0.30 mL of starch solution (1%, substrate) in sodium phosphate buffer (0.02 M, pH = 6.9 with 6 mM NaCl) was added, and the mixture was further incubated at 37 °C for 20 min. Subsequently, it was mixed with 0.20 mL of dinitrosalicylic acid reagent before being kept in a boiling water pot for 5 min and cooled down to room temperature. The cooled mixture was then diluted with 10 mL of distilled water, and its absorbance was measured at 540 nm using a spectrophotometer (Shimadzu, Japan).

To test α-GIA, 0.1 mL of the sample (2 mg protein/mL) was incubated with 0.1 mL α-glucosidase (1 U/mL) in 0.01 M phosphate buffer (pH = 7.0) and 2.2 mL of 0.01 M phosphate buffer (pH = 7.0) at 37 °C for 5 min. Then, 0.1 mL of pNPG (3 mM, substrate) in 0.01 M phosphate buffer (pH = 7.0) was added to the mixture and was further incubated at 37 °C for 30 min. The reaction was stopped by adding 1.50 mL of 0.1 M Na_2 CO_3 solution before recording the absorbance at 405 nm by employing the spectrophotometer [36].

The α-AIA and α-GIA of the protein hydrolysate were calculated as follows:

$$\alpha - \text{AIA or } \alpha - \text{GIA}(\%) = \frac{(A_1 - A_2) - (A_3 - A_4)}{A_1 - A_2} \times 100 \quad (1)$$

where A_1 is the absorbance of the control (the mixture containing enzyme, substrate, and no hydrolysate or peptide fraction); A_2 is the absorbance of the control blank (the mixture containing substrate, no enzyme, and no hydrolysate or peptide fraction); A_3 is the absorbance of the tested sample (the mixture containing enzyme, substrate, and hydrolysate or peptide fraction); A_4 is the absorbance of the sample blank (the mixture containing hydrolysate or peptide fraction, substrate, and no enzyme).

Logarithmic regression analysis was employed to determine the half inhibitory concentration (IC_{50}, the concentration of the inhibitor to inhibit 50% of enzyme activity) for α-AIA and α-GIA of the earthworm protein hydrolysates and their 5 peptide fractions.

Acarbose was used as the positive control with a concentration range of 50–250 (μg/mL) for the α-AIA test and 200–1000 (μg/mL) for the α-GIA test.

The effects of hydrolysis conditions on the α-AIA and α-GIA of the earthworm protein hydrolysate were investigated. The effects of 6 factors, including hydrolysis enzyme type, earthworm: phosphate buffer ratio, temperature, pH, enzyme: substrate (E:S) ratio, and hydrolysis time, were assessed to obtain the best condition that generates hydrolysate with the highest activity. After that, each collected hydrolysate was fractionated to gain its peptide fractions.

2.2.4. Fractionation of Earthworms Protein Hydrolysate

By using the ultrafiltration centrifugal devices of 30 kDa, 10 kDa, 3 kDa, and 1 kDa (Macrosep, Pall Laboratory, New Port Richey, FL, USA), the earthworm protein hydrolysate was further fractionated. Five peptide fractions (<1 kDa, 1–3 kDa, 3–10 kDa, 10–30 kDa, and >30 kDa) of each hydrolysate were obtained and their α-AIA and α-GIA were evaluated.

2.2.5. In Vitro Digestion

The in vitro digestion test of the earthworm protein hydrolysates and their peptide fractions was conducted following the method described in Kang et al. [37]. The sample, with a protein concentration of 5% (*w*/*v*), was adjusted to pH = 2 using 6 M HCl solution, and its temperature was increased to 37 °C. Pepsin was then added with an E:S ratio of 4% (*w*/*w*), and the mixture was further incubated at 37 °C for 1 h and shaken at 230 rpm. Subsequently, its pH was changed to 7.5 by utilizing 6 M NaOH solution before adding pancreatin with an E:S ratio of 4% (*w*/*w*). The mixture was continuously incubated at 37 °C and shaken at 230 rpm for 2 h. The enzymes were inactivated by heating the mixture at 90 °C for 10 min. After that, the sample was collected to determine its α-AIA and α-GIA.

2.2.6. Thermal and pH Stability

The method described in Sripokar et al. [38] was used to evaluate the thermal and pH stability of the protein hydrolysates and their peptide fractions.

To assess the thermal stability, 5 mL of the sample (40 mg protein/mL) was heated at 100 °C for 15, 30, 45, 60, 90, 120, 150, and 180 min. After that, the solution was cooled down to room temperature in iced water and diluted to the final volume of 10 mL with deionized water, and its α-AIA and α-GIA were measured.

In order to examine the pH stability, the pH of 5 mL of the sample (40 mg protein/mL) was adjusted to 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 using 6 M HCl or 6 M NaOH solution and placed at room temperature for 30 min. The sample was then altered to pH = 7.0 by employing 1 M phosphate buffer and diluted to the final volume of 20 mL with deionized water, before performing the α-AIA and α-GIA assays.

Both the thermal stability and pH stability of the sample were expressed as the relative activity (%), which was defined as the percentage of bioactivity of the treated sample compared to that of the control (untreated sample).

2.2.7. Statistical Analysis

All experiments were performed in triplicate. The data presented as mean ± standard deviation were processed using the software Excel. The statistically significant differences were determined using the software SPSS (IBM SPSS Statistics 20).

3. Results and Discussion

3.1. Investigation Results of α-AIA and α-GIA of the Earthworm Hydrolysate under the Influence of Hydrolysis Condition

3.1.1. Effect of Hydrolysis Enzyme

Peptides' bioactivity is significantly associated with their amino acid composition and sequence determined by the specificity of the enzymes employed in the hydrolysis process [39].

Regarding α-AIA, Protamex was the most suitable protease among the four enzyme preparations to acquire the earthworm protein hydrolysate, with the highest α-AIA of 73.91 ± 1.45% (Figure 1a). The enzyme was known to be a mixture of endoproteases and exopeptidases [40,41], which offered a broad specificity for aliphatic amino acids [42]. In addition, it produced peptides with a high frequency of Leu, Glu, Lys, Met, Val, and Arg at the C-terminal and Lys, Gly, Ala, Ile, His, Asp, and Phe at the N-terminal [43,44], fitting to the characteristics of α-amylase inhibitory peptides in the literature. For instance, the results of Baba et al. [7] showed that most α-amylase inhibitory peptides contained Leu and Met at their N- and C-ends. In addition, Balderas-León et al. [45] have emphasized the roles of hydrophobic (Ala, Leu, Val, and Gly) and hydrophilic amino acids (Met and His) in the α-AIA of the containing peptides [19]. Furthermore, the α-AIA of peptides was enhanced with the presence of Gly, although the exact mechanism was unknown [46]. Moreover, α-amylase has aromatic residues lying within the substrate-binding pocket and the aromatic-aromatic interactions seem to be predominantly involved in the α-AIA of peptides containing Phe [46]. In addition, Arg, Glu, and Asp were interpreted to

own the highest α-amylase inhibitory effects with their binding energies of −5.2, −5.0, and −4.8 kcal/mol, respectively [11]. Additionally, the molecular docking results in Siahbalaei et al. [11] revealed that Glu and Ala could be bound to the allosteric binding site of α-amylase at its Arg398, Arg421, Asp402, Arg252, Glu23, Glu863, and Arg437, changing its conformation and thus diminishing its catalysis function. Protamex was used to obtain the *Paeonia ostii* cake protein hydrolysate with the highest α-AIA as well [46].

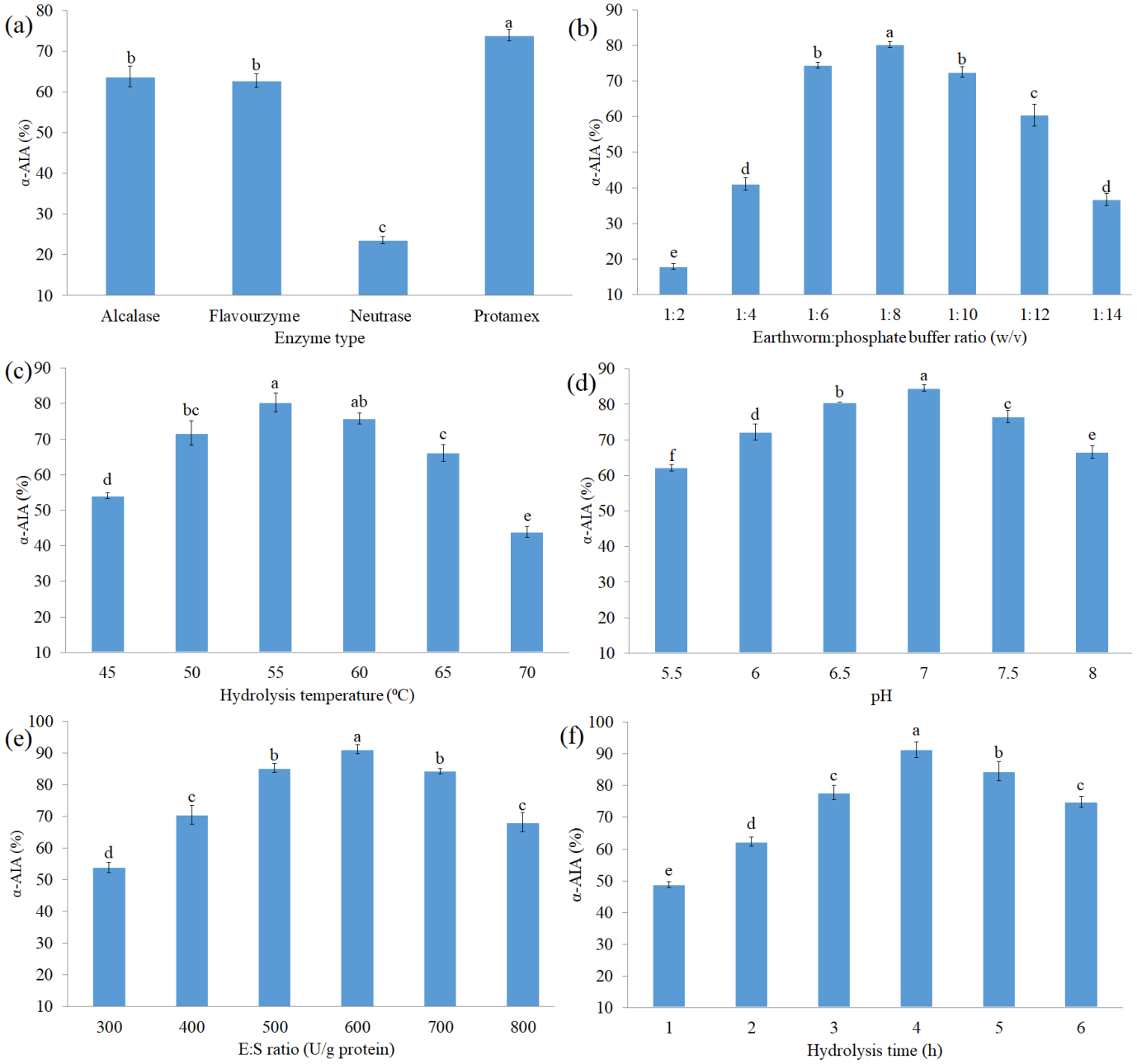

Figure 1. Effect of hydrolysis enzyme type (**a**), earthworm: phosphate buffer ratio (**b**), temperature (**c**), pH (**d**), enzyme: substrate (E:S) ratio (**e**), and hydrolysis time (**f**) on the α-amylase inhibitory activity (α-AIA) of the earthworm protein hydrolysate. The bars with different letters indicate the significant differences ($p < 0.05$).

As for α-GIA, the hydrolysate of earthworm protein by Alcalase exhibited the highest activity of 37.66 ± 0.62% (Figure 2a). Alcalase (an endopeptidase) has a preferential cleavage of peptide bonds when the polypeptide chain contains amino acids Glu, Met, Leu, Tyr, Lys, Thr, Ile, Phe, Arg, and Gln [39,43]. In addition, it usually releases the

peptides with N-terminals of Gly, Lys, Arg, Asp, Ala, Trp, and Ile [43]. These properties of the Alcalase-generated peptides were mostly similar to those of α-glucosidase inhibitory peptides published previously. Three α-glucosidase inhibitory peptides identified in the study of Feng et al. [47] had their N-terminals of Gly and C-terminals of Lys and Arg. Lys and Arg occupying the N-terminal of the peptide also appeared to greatly influence the α-glucosidase inhibition [8]. Similarly, Met located at any terminal ends was found to play a key role in peptides with α-GIA [7,8]. On the other hand, the contribution of hydrophobic amino acids, such as Phe, Leu, Ile, and Ala to α-GIA of the containing peptides, could not be belittled [6,48]. Additionally, Asp and Gln were demonstrated to attach to the α-glucosidase's allosteric binding site through molecular docking analysis [10]. Alcalase was also the best choice to obtain the α-glucosidase inhibitory hydrolysates from various protein sources [25,47,49,50]. Therefore, Protamex and Alcalase were correspondingly chosen for further investigations on the α-AIA and α-GIA of the earthworm protein hydrolysates.

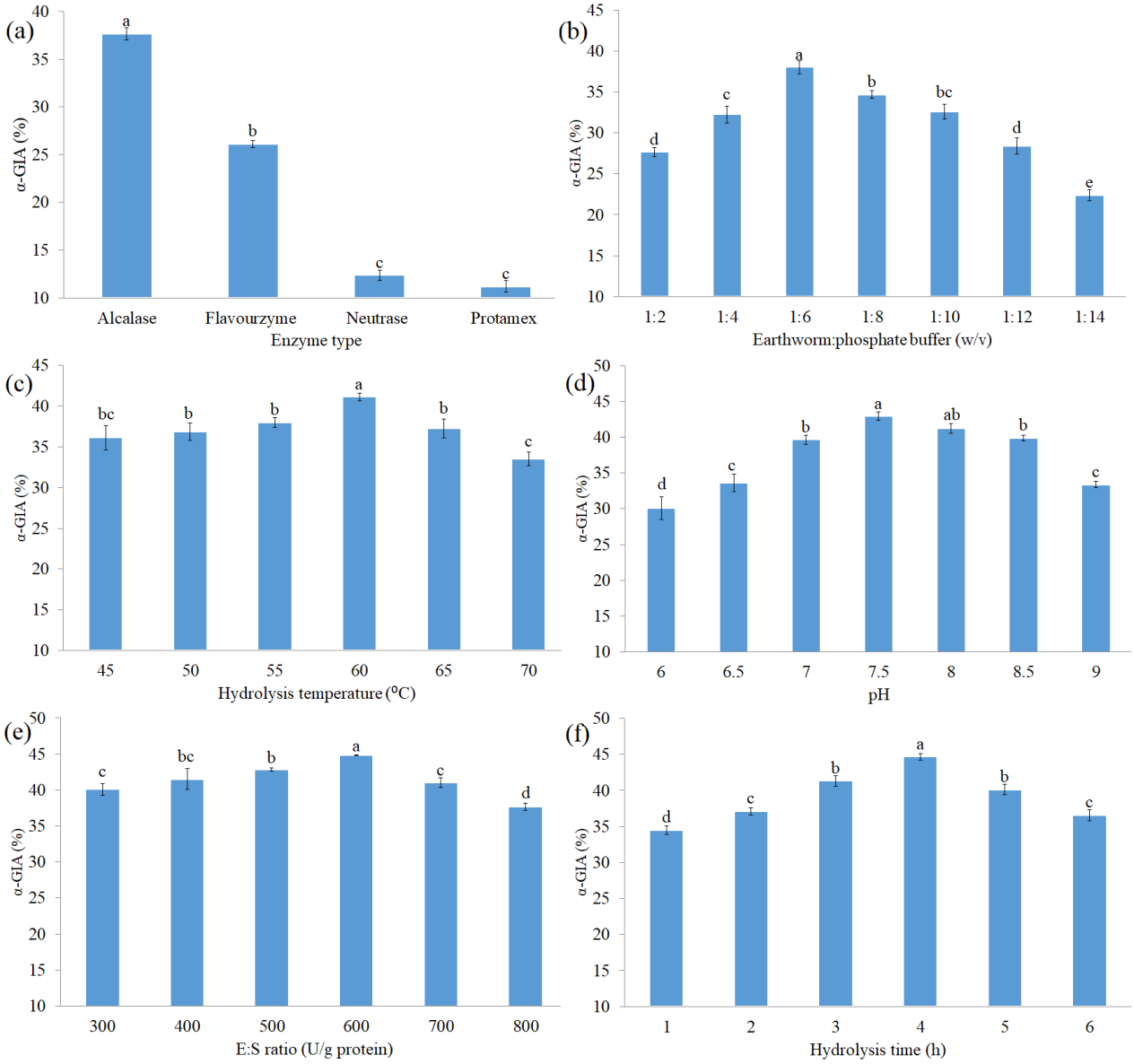

Figure 2. Effect of hydrolysis enzyme type (**a**), earthworm: phosphate buffer ratio (**b**), temperature (**c**), pH (**d**), E:S ratio (**e**), and hydrolysis time (**f**) on the α-glucosidase inhibitory activity (α-GIA) of the earthworm protein hydrolysate. The bars with different letters indicate the significant differences ($p < 0.05$).

3.1.2. Effect of Earthworm: Phosphate Buffer Ratio

A large amount of buffer can reduce the probability of collisions between the substrate and enzyme molecules, obstructing the production of bioactive peptides from the intact protein and lowering the bioactivity of the protein hydrolysate [51,52]. In contrast, high viscosity is a major challenge for enzymatic hydrolysis at a small amount of buffer. It limits the diffusion rate of protein molecules, and thereby, protein aggregation easily happens, which inhibits the accessibility of the enzyme [51]. On the other hand, a sufficient amount of buffer not only provides high protein contents in the hydrolysate but also quickly scatters the products of hydrolysis, preventing the feedback effect and enhancing the bioactivity of the protein hydrolysate [52]. In this study, Figures 1b and 2b indicate that α-AIA and α-GIA of the hydrolysates reached their peaks at the earthworm: phosphate buffer ratios of 1:8 (w/v) and 1:6 (w/v), respectively, which were the selected levels for further investigations.

3.1.3. Effect of Hydrolysis Temperature

As illustrated in Figures 1c and 2c, the highest α-AIA of 80.30 ± 2.62% and α-GIA of 41.13 ± 0.50% were observed at 55 °C and 60 °C, respectively. At low temperatures, the protein and enzyme molecules own low kinetic energy, lessening the formation rate of the enzyme-substrate complex, and thus, a low content of bioactive peptides is generated in the hydrolysate [33,51]. However, irreversible denaturation of active catalyst molecules would occur at high temperatures, diminishing the generation of peptides with α-AIA and α-GIA [53]. Hence, 55 °C and 60 °C were set for further studies.

3.1.4. Effect of pH

The charge distribution and conformation of both substrate and enzyme molecules are remarkably affected by the environmental pH, influencing enzyme-substrate assemblies and the bioactivity of the protein hydrolysate [52,54]. In addition, pH could either enable or disable the solubility of protein molecules by changing their ionization ability and ameliorating or attenuating the hydrolysis process, altering the α-AIA and α-GIA of the hydrolysate [55]. Furthermore, under unfavorable pH, the specific spatial structures of the enzyme would be disrupted, and thus, the enzyme function will be negatively influenced [51,56]. In this study, the suitable pH for the earthworm protein hydrolysis to gain the hydrolysates with the highest α-AIA and α-GIA were 7.0 and 7.5, respectively.

3.1.5. Effect of E:S Ratio

Both α-AIA and α-GIA of the earthworm protein hydrolysates were in direct proportion to the E:S ratio within the range of 300–600 U/g protein, and then, their relation became an inverse proportion in an E:S ratio range from 600 to 800 U/g protein (Figures 1e and 2e). It is a common observation that could be found in many studies on the relationship between the E:S ratio and bioactivity of the obtained protein hydrolysates [33,54,55]. A popular explanation for this case is that with the increase in enzyme amount, protein hydrolysis proceeds more swiftly to create bioactive peptides, although they could be further degraded if a very high enzyme dosage was used [51]. Therefore, 600 U/g protein was applied in the following experiments.

3.1.6. Effect of Hydrolysis Time

Figures 1f and 2f indicate that α-AIA and α-GIA of the earthworm protein hydrolysates increased up to a threshold of hydrolysis time of 4 h and declined afterward. This could be due to the fact that in the first period of hydrolysis, the enzyme disrupts the quaternary, tertiary, and secondary structure of the intact earthworm proteins to release peptides with high α-AIA and α-GIA into the hydrolysate [9,55]. An extensive hydrolysis time, however, may trigger the degradation of these bioactive peptides, decreasing the bioactivities [53]. A similar pattern of α-AIA and α-GIA in relation to the hydrolysis time was reported by Mojica and Mejía [49] and Kang et al. [37]. Therefore, the hydrolysis time of 4 h was chosen for further analysis.

3.2. α-AIA and α-GIA of Peptide Fractions

As seen in Figure 3, the lower the molecular weight of the peptide fraction was, the greater its α-AIA and α-GIA were. The finding in this study was in agreement with those of several studies [47,57,58]. Probably, the low-molecular-weight peptides can straightforwardly attach to the active site of the α-amylase or α-glucosidase, changing the enzyme's configurations and blocking their substrate-binding sites [57,58]. In addition, Zhu et al. [9] reported that small peptides are more flexible than large peptides, and they can bind to the enzyme-substrate complex, causing an uncompetitive inhibition phenomenon and reducing the enzyme's activity. The high flexibility of small peptides also promotes the hydrophobic, hydrogen bonding, and electrostatic interactions with starches (substrate of α-amylase), hindering the formation of enzyme-substrate complex [9]. In contrast, the sterical effect is the main problem that disrupts the attachment of large peptides to the enzymes' catalytic sites, lessening their α-AIA and α-GIA [47,59]. In our study, the <1 kDa peptide fraction expressed the highest α-AIA and α-GIA (Figure 3). Nevertheless, the highest α-AIA was found at the >10 kDa fraction from the lima bean hydrolysate [57] and at the 3–5 kDa fraction from the yellow field pea hydrolysate [60]. Meanwhile, the 5–10 kDa fraction of the lima bean hydrolysate and the 1–3 kDa fraction of the yellow field pea hydrolysate displayed the greatest α-GIA [57,60]. The difference in the peptide composition in various protein sources and used hydrolysis enzyme preparations may cause the dissimilarity in the findings of these different publications [57,60].

In terms of the inhibition potential of the earthworm protein hydrolysates/peptide fractions, the α-AIA of the <1 kDa fraction was 335.42, 3.06, and 5.47 times greater than those of the >10 kDa peptide fraction from the lima bean hydrolysate [57], *Scutellaria baicalensis* shoots extract [61], and *Persicaria hydropiper* L. leaves essential oils [62], respectively. Meanwhile, its α-GIA was 390.18 and 1.82 folds higher than those of the 5–10 kDa peptide fraction from the lima bean hydrolysate [57] and *Scutellaria baicalensis* shoots extract [61], respectively. In addition, the <1 kDa peptide fraction in our study showed higher α-AIA (its IC50 being 4.05 times lower) and lower α-GIA (its IC50 being 1.86 folds higher) than that of Acarbose, a common antidiabetic drug (Figure 3). However, its α-GIA was approximately the same as that of voglibose, a reversible α-glucosidase inhibitor (IC50 of 310 μg/mL) [63].

For the action mechanism of the protein hydrolysates/peptide fractions, peptides were known to inhibit these enzymes by binding to either enzyme's catalytic sites to block the access of substrate molecules or to other sites on the enzymes to alter their conformation [27]. The interactions between the peptides and enzymes could comprise aromatic-aromatic interactions, hydrogen bonds, electrostatic, and Van der Waals interactions [46]. Similarly, acarbose (a common antidiabetic drug) inhibits α-amylase or α-glucosidase by competing with the substrates for the enzymes' active sites [64]. In the same way, phytochemicals in plant-based extracts or essential oils attach to α-amylase or α-glucosidase through aromatic-aromatic interactions, hydrogen bonds, and Van der Waals interactions that are similar to those between peptides and the enzymes, except for the electrostatic interactions [65,66].

The specificity of the protein hydrolysates/peptide fractions for α-amylase and α-glucosidase was mainly affected by their conformations [67]. Their high specificity and affinity to the enzymes were also reported in Ibrahim et al. [68] and Chelliah et al. [26]. In a similar way, Acarbose (a pseudo-tetrasaccharide consisting of maltose bridged to acarvosine) displays an excellent specificity for these starch-digestive enzymes [23] by mimicking the substrate structure [68].

Therefore, the <1 kDa peptide fraction from the earthworm protein hydrolysate could be considered a potential antidiabetic agent.

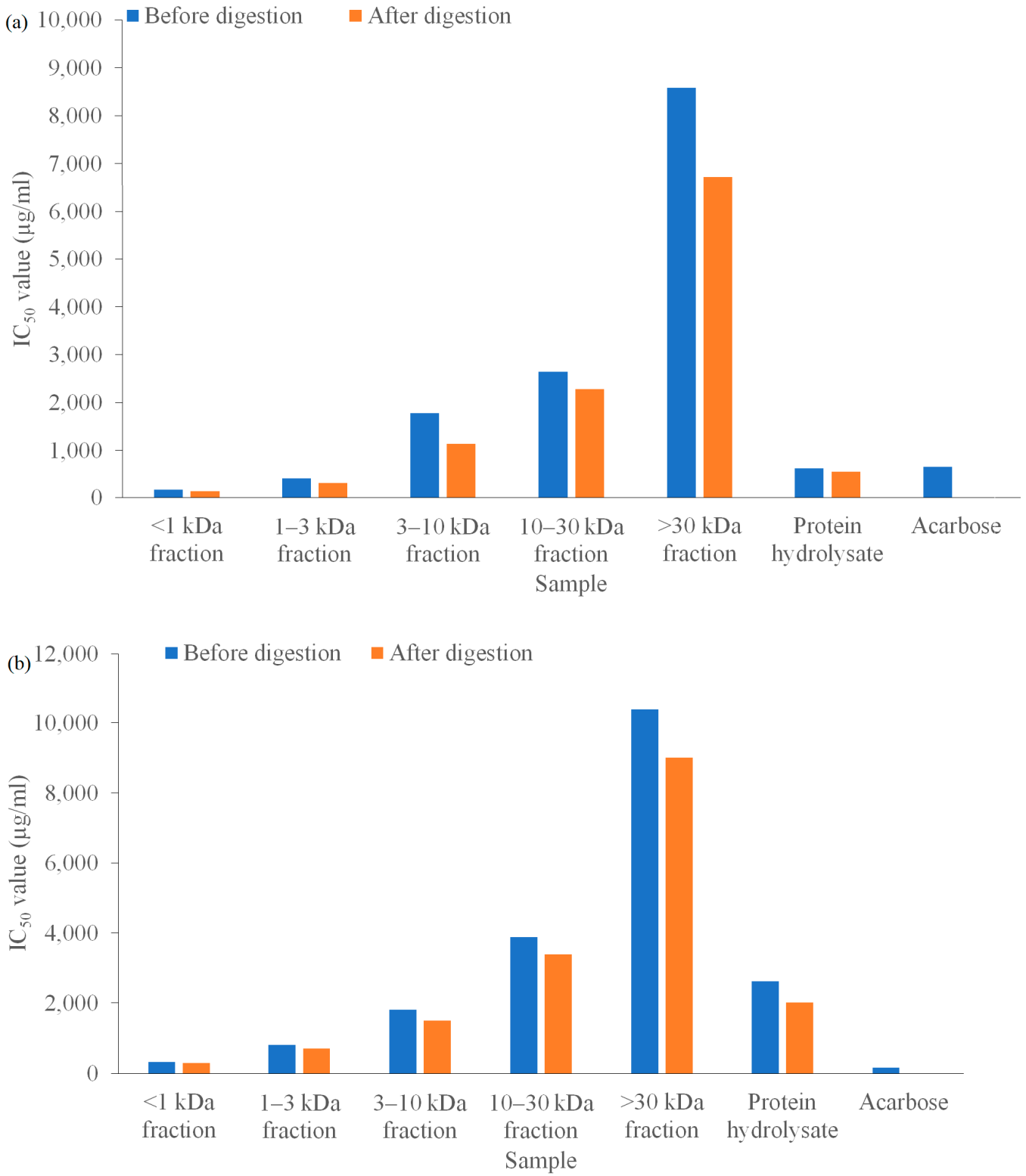

Figure 3. α-AIA (**a**) and α-GIA (**b**) expressed by the half inhibitory concentration (IC_{50}) (μg/mL) of the earthworm protein hydrolysate, their 5 peptide fractions, and the positive control (Acarbose) before and after the simulated digestion.

3.3. Investigation Results of In Vitro Digestion Stability of α-AIA and α-GIA of the Hydrolysates and Their Peptide Fractions

For exploitation as an α-amylase or α-glucosidase inhibitor, the bioactive peptides must reach their targets (α-amylase and α-glucosidase), which mostly act in the small intestine [8,69]. This could be achieved if the peptides tolerate harsh acidic conditions and with digestive enzymes in the gastrointestinal tract. The bioactivity of the protein hydrolysates/peptides after in vitro digestion seems to be a good indicator of their bioavailability in the human body [70]. In this study, after the in vitro digestion test, the α-AIA of the earthworm hydrolysate and its 5 peptide fractions enhanced from 1.15 to 1.56 folds, whereas the α-GIA of the tested samples increased from 1.15 to 1.29 times. It could be explained by several hypotheses. Firstly, the active sequence of the peptides would not be degraded by digestive enzymes [71]. Secondly, some peptides could resist gastrointestinal peptidases, especially for short-chained peptides and Pro-containing peptides [71,72]. Thirdly, Qiao et al. [46] and Zhu et al. [9] proposed that the hydrolysed proteins from pepsin-pancreatin could enhance the amount of V-type resistant starch, restraining the activity of α-amylase. In addition, the newer short peptides generated by pepsin and pancreatin were reported to express higher α-AIA and α-GIA than the initial peptides [72,73]. Similar observations could be found in the studies of Mojica and Mejía [49], Ninomiya et al. [74], and Wang et al. [75]. The result indicated that the earthworm protein hydrolysates and their 5 derived-peptide fractions could be potent α-amylase or α-glucosidase inhibitors.

3.4. Investigation Results of Thermal and pH Stability of α-AIA and α-GIA of the Hydrolysates and Their Peptide Fractions

The stability of the hydrolysate and peptide fractions towards thermal and pH treatments (two popular food treatments) should be taken into account as a prerequisite for incorporating them into food or nutraceutical products [76].

As seen in Figure 4, the stability of α-AIA and α-GIA of the hydrolysate and 5 peptide fractions reached the peaks at pH = 7 and 8. It is acknowledged that most proteins are stable at around neutral pH because of the low net electrostatic repulsive energy, which minimizes the swelling and unfolding of protein molecules, and thereby, remaining their bioactivity [77]. Nevertheless, the α-AIA and α-GIA stability of these samples gradually decreased as the pH shifted to very low or very high values (Figure 4). This could be due to the changes in the charges of peptides, particularly at the N- and C-terminus at high acidic and alkaline conditions [78], altering the electrostatic interactions and hydrogen bonds and transforming their secondary structures and solubility, resulting in the loss of bioactivity [79,80]. The change in the stability of these samples could be due to the impact of pH on one particular or more amino acids. For instance, Gln and Arg were vulnerable under acid treatment while alkaline conditions could destroy Cys, Ser, and Thr [71]. In addition, deamination and racemization reactions under alkaline conditions could take charge of the decrease in the α-AIA and α-GIA stability of the samples at high pH values [72,81].

On the other hand, temperature is considered the most common factor impacting protein stability. High temperatures not only break down the noncovalent interactions (hydrophobic interactions, electrostatic interactions, and hydrogen bonds) but also enhance the conformational entropy of the protein molecules, causing protein destabilization and losing their bioactivity [77]. In this study, in the first 15 min of heat treatment, the α-AIA and α-GIA of all samples remained unchanged (Figure 5). This could be due to the presence of high contents of peptides containing specific amino acids that enhanced their thermal stability. For example, Ile assists in greatly bundling up the interior core of the protein, reducing its void spaces. Consequently, the mobility of the polypeptide chain at high temperatures decreased, minimizing its conformational entropy and contributing to stabilizing protein/peptide at high temperatures [77]. Val, Leu, and Phe were also found to have the same role as Ile in protein stability [82]. However, the bioactivity stability of these samples progressively diminished as heating time was continuously prolonged to 180 min

(Figure 5). The observation was similar to the previous publication of Wali et al. [81] that with different heating times, the bioactivity of peptides declined after the threshold value. The reason behind this could be attributed to protein denaturation, aggregation, and amino acid degradation. High temperatures break down the intermolecular and intramolecular interactions, exposing hydrophobic domains and causing protein aggregation [79]. It also resulted from the random reformation of intramolecular disulphide bonds [74]. Simultaneously, the deamination of Asn and Gln, as well as oxidation of Cys, Met, and Trp at high temperatures, lowered the α-AIA and α-GIA stability of the protein hydrolysate and peptide fractions [77].

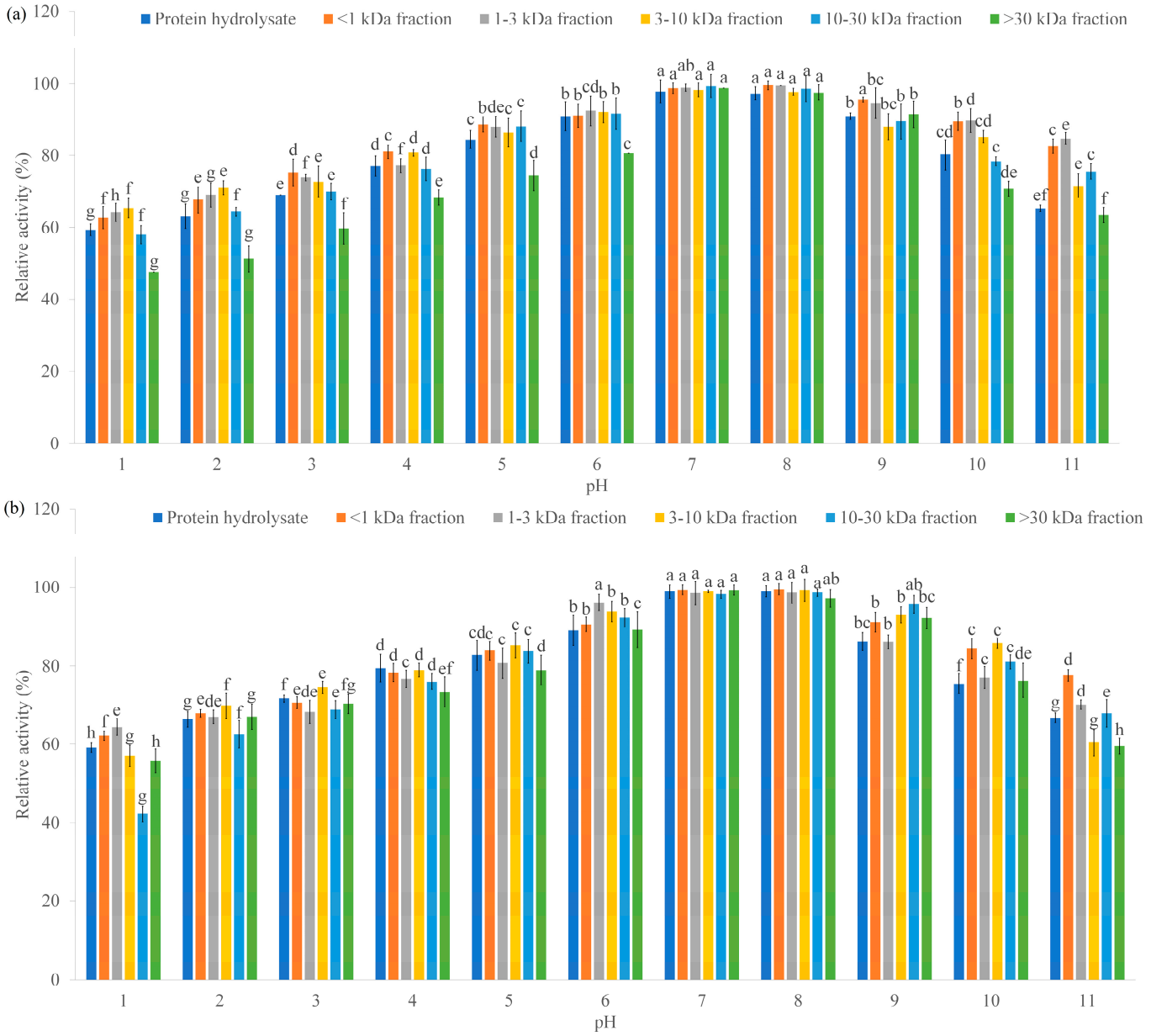

Figure 4. pH stability of α-AIA (**a**) and α-GIA (**b**) of the earthworm protein hydrolysate and their 5 peptide fractions. The same color bars with different letters indicate the significant differences ($p < 0.05$).

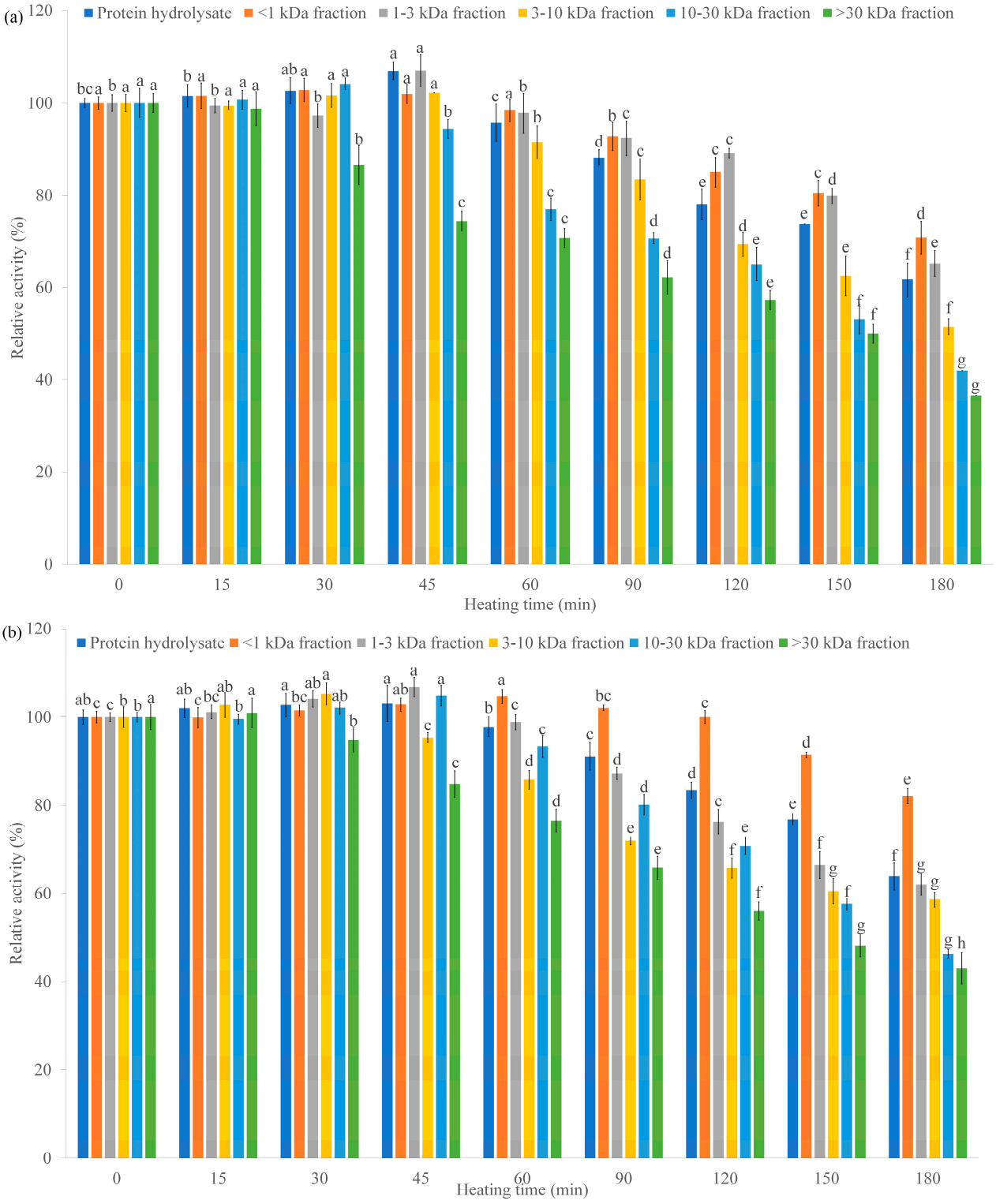

Figure 5. Thermal stability of α-AIA (**a**) and α-GIA (**b**) of the earthworm protein hydrolysate and their 5 peptide fractions. The same color bars with different letters indicate the significant differences ($p < 0.05$).

Regarding the peptide molecular weight, an inverse proportion between the peptide molecular weight and thermal stability was observed (Figure 5). This agreed with the findings of Klomklao and Benjakul [79] that peptides with smaller sizes are more stable to aggregation at high temperatures than the larger peptides. López-Sánchez et al. [71] also

stated that large peptides easily form clusters which may prevent them from binding to the active site of the enzymes, reducing their α-AIA and α-GIA.

In general, the earthworm protein hydrolysates and their 5 peptide fractions remained over 40% of α-AIA and α-GIA after wide-range pH treatment or heat treatment at 100 °C for 180 min. It comes to a suggestion that these samples can be applied to a broad range of food or nutraceutical products.

4. Conclusions

This study has discovered a new bioactivity (antidiabetic activity) of the protein hydrolysates/peptide fractions derived from the earthworm. Especially, the <1 kDa peptide fraction of the hydrolysate showed a higher α-AIA than that of Acarbose, with approximately the same α-GIA as that of voglibose. Thus, the protein hydrolysates/peptide fractions could act as potential α-amylase or α-glucosidase inhibitors, being fortified to a broad range of functional foods or nutraceutical products, supporting diabetes treatment and reducing the utilization of synthetic drugs and their side effects. However, further characterization of the peptides, identification of their active components and potential mechanisms of the action of the peptides within a living organism, in vivo tests, allergy assays, or clinical trials, should be done to strengthen their application probability in reality.

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