ORIGINAL ARTICLE

Antihyperglycaemic Efficacy of Black Soldier Fly (BSF) Larval Protein Hydrolysate as a Future Antidiabetic Candidate

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ABSTRACT

Introduction: Hyperglycaemia can occur because of decreased insulin secretion due to pancreatic dysfunction or insulin resistance. The different amino acid content of black soldier fly (Hermetia illucens) larvae can protect pancreatic beta cells from oxidative stress in patients with diabetes. Protein hydrolysate is a product of protein breakdown catalysed by enzymes into smaller peptides. Insulinotropic amino acids have several pathways that induce insulin synthesis. **Methods:** Sample preparation of larval protein hydrolysate through dissolution, homogenisation, sonication, enzyme digestion, centrifugation, and lyophilisation. Identify amino acids using LC-MS/MS by dissolving the hydrolysate and ionising the molecules to be separated by the mass analyser. The results were interpreted using the MassLynx software. The α-glucosidase inhibitory assay was conducted for hydrolysate with trypsin incubated for 7 hours. An oral glucose tolerance test was conducted to evaluate hypoglycaemic ability. The selected protein hydrolysate and sham controls were fed orally to male Sprague–Dawley rats using oral gavage. **Results:** The selected larval protein hydrolysate was a trypsin catalyst incubated for 7 hours. The LC-MS/MS results showed that the chosen protein hydrolysate was based on the types of insulinotropic amino acids, including lysine, methionine, histidine, proline, and ornithine. Hydrolysate insignificantly inhibits glucose absorption in the intestine through the α-glucosidase enzyme. The selected dose was 300 mg/kg BW. **Conclusion:** The selected hydrolysate was hydrolysed by trypsin enzyme for 7 hours, with an optimum dose of 300 mg/kg BW, to produce the desired hypoglycaemic effect.
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INTRODUCTION

Diabetes mellitus (DM) is a degenerative and non-communicable disease characterised by hyperglycaemia as compensation for chronic metabolic disorders (1). DM is classified into two types, i.e. DM type 1 and 2 (2). Type 1 DM is an autoimmune disease triggered by the degeneration of pancreatic beta cells due to an immunogenic disorder in the human leukocyte antigen (HLA) gene allele in major histocompatibility complex (MHC) class II (3). Meanwhile, type 2 DM is a clinical manifestation of an unhealthy lifestyle that leads to obesity and insulin resistance (4). Diabetes is one of the foremost contributors to mortality among non-communicable diseases. The peril of DM-induced complications triggered by sustained hyperglycaemia cannot be overstated, as it can lead to many life-threatening degenerative conditions, including kidney and heart failure (5). These complications often affect critical organs, such as the brain, lungs, and heart, and all these organs depend on the circulatory system's homeostasis (6). Hyperglycaemia in DM is responsible for circulatory system imbalance due to the excess production of reactive oxygen species (ROS) induced by oxidative stress, stimulating atherosclerosis formation and leading to complications (7).

Approximately 537 million people aged 20–79 have coexisted with diabetes. This number continues to increase and is predicted to reach 643 million individuals in 2030 and 783 million in 2040 (8). The epidemiology of diabetes mellitus shows an increasing trend globally, especially in developing countries such as Indonesia. In 2021, the International Diabetes Federation (IDF) reported that Indonesia ranks fifth globally, with around 19.5 million people with diabetes (8). This disease also ranked ninth as a cause of death in 2019, with 1.5 million cases (1, 9). Based on the predictions that have been reported, the DM threat is a critical problem that must be resolved immediately. Therefore, biomedical innovations and products are needed to prevent or treat DM-induced complications.

Biomedical innovations as candidates for antidiabetic medicine are expected to have antihyperglycemic properties with minimal side effects. These properties can be found in natural compounds, such as primary and secondary metabolites (10), which promote tissue repair and stimulate as well as inhibit biochemical reactions (11). In this regard, one organism currently becoming a trend in biomedical research is the black soldier fly (BSF) larvae (Hermetia illucens). BSF larvae had a high protein content of approximately 53% (12). BSF protein is a macromolecule comprising primary metabolites, amino acids, that are thought to have unique functions in the human body, mainly as promoters in biochemical reactions such as stimulating hormonal secretion (13).

An example of a hormone that can be modulated by free amino acids and is related to this study is insulin; therefore, amino acids with insulin-stimulating properties are referred to as insulinotropic (14). The insulinotropic ability of free amino acids in BSF larvae, such as lysine, histidine, arginine, and several others, is mediated through various pathways involving the tricarboxylic acid (TCA) cycle, plasma membrane depolarisation, and sodium ion co-transport (15). Additionally, insulinotropic amino acids such as histidine and valine are categorised as antioxidants because they can scavenge free radicals. This ability can protect beta cells from damage stimulated by oxidative stress (16, 17). In addition, hydrophobic amino acids, such as leucine, are known to have antioxidant capabilities and can inhibit the α-glycosidase enzyme (18). The ability to inhibit this enzyme is essential for antidiabetic drug candidates because it can suppress the absorption of blood glucose from the intestine, similar to the effect of the commercially available medicine, acarbose.

Therefore, BSF was chosen as an alternative raw material for manufacturing antidiabetic drug candidates via an enzymatic hydrolysis process. This study aimed to obtain the best larval protein hydrolysate with the most insulinotropic amino acids. Subsequently, the selected larval protein hydrolysate was tested for its efficacy through in vitro and in vivo studies utilising α-glucosidase inhibitory assay and oral glucose tolerance tests in hyperglycaemia model rats to determine the optimal dose.

MATERIALS AND METHODS

Samples

BSF larvae were obtained from PT. Biomagg Sinergi Internasional (Depok, Indonesia) and directly brought to the Biochemistry Laboratory (Faculty of Mathematics and Natural Sciences, IPB University) inside a cool box to maintain the cold-chain process. Sample preparation was referred to the hydrolysis methods from Khesal et al. (19) with modifications to produce protein hydrolysate. Prior to hydrolysis, sterilise all equipment using an autoclave (TOMY ES-215; TOMY, Tokyo, Japan). The BSF larvae were blended using a homogeniser T25 Ultra-Turrax (IKA Works, Ohio, USA) for 2 minutes, adding a phosphate buffer of pH 7.5. The larval mixture was homogenised for 2 minutes and heated for 15 minutes at 98°C in a water bath SYK-382-M (Sanyo Rikagaku Kikai, Japan). The samples were then sonicated in a bath sonicator (Decon Laboratories, Hove, UK) for 45 minutes to break into smaller bonds.

Furthermore, 8.8 mg of trypsin enzyme was dissolved in 0.1M phosphate buffer of pH 8.0. The dissolved enzyme is mixed with the formerly sonicated sample. The hydrolysate was incubated using trypsin in a WiseBath 18L water-bath shaker (Daihan Scientific, Gangwon, South Korea) at 37°C following three different incubation times, including 3, 5, and 7 hours. After incubation, the enzymatic activity was deactivated by heating the samples at 85°C for 15 minutes. The hydrolysates were allowed to cool, weighed, and centrifuged using a Beckman Microfuge 11 (Beckman Instruments, Palo Alto, California) at 4°C for 15 minutes (13,000 g). The supernatant was subsequently freeze-dried using a BK-FD10P Tabletop Freeze Dryer (Biobase Laboratory, Shandong, China) for two days.

Identification of amino acids using LC-MS/MS

The BSF larval protein hydrolysates were sent to the Indonesian National Police Forensic Laboratory Centre to identify the levels and types of free amino acids in each sample. The hydrolysates were separated using an Acquity UPLC® HSS C18 column (1.8 µm, 21x100 mm, Waters, USA) with a G2Q/TOF micromass spectrometer (Waters, USA) and an electrospray ionisation (ESI) source at 50°C. The samples (5 µl) were injected and run for 23 minutes at a 0.2 ml/min flow rate. The mobile phase consisted of solution A (water + 5 mM ammonium formic) and solution B (acetonitrile + 0.05% formic acid). The analysis was conducted with a full scan at 50–1300 m/z. Ionisation was performed in the positive ESI mode. The mass spectrometers were operated under the following conditions: 4-volt collision energy, 25–50-volt colt ramp, 100°C temperature source, 350°C desolvation temperature, and desolvation gas flow at 793 l/h. The data obtained from the UPLC-ESI-QTOF was processed using MassLynx V4.1 software. The molecular masses of the amino acids of interest were matched with the molecular mass reference from the PubChem database (PubChem https://pubchem.ncbi.nlm.nih.gov/).

α-glycosidase inhibitory assay

Selected BSF larval protein hydrolysates were tested in vitro through the α-glucosidase inhibitory assay at the laboratory of Bogor Biopharmaceutical Study Centre. The test method was referred to Etsassala et al. (20) with modification. The first procedure was to prepare an enzyme solution; 1 mg of α-glucosidase enzyme was dissolved in 0.01 M phosphate buffer (pH 7.0) containing 200 mg of serum bovine albumin. Before continuing to the next step, 1 ml of the enzyme solution

was diluted 25 times with the phosphate buffer. The BSF larval protein hydrolysate was dissolved in 1, 1.5, and 2% (w/v) dimethyl sulfoxide. The reagent mixture was α-glucosidase enzyme and substrate, which were incubated at 37 ℃ for 30 minutes and inactivated with Na2CO3, then measured with a microplate reader at a maximum wavelength of 410 nm. The hydrolysate and enzyme mixture (A) was used to correct the hydrolysate absorbance. Sham control (B) used a mixture without hydrolysate. A positive control was prepared by dissolving acarbose in a phosphate buffer. The solutions were centrifuged, and the supernatant was collected and mixed with the reagent. The reaction results were measured using a spectrophotometer at a wavelength of 410 nm. A positive control was used for comparison, and the sample was tested three times. For each sample, the percentage inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = 1 - \frac{\text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

Hypoglycaemic test with oral glucose tolerance test (OGTT)

Approval and ethical clearance from the Animal Ethics Commission of IPB University were attained upon commencement of the study (Reference No: 135/KEH/SKE/V/2019). The animal models of hyperglycaemia were male Sprague–Dawley rats (Bogor Veterinary Research Centre, Indonesia). Male rats were chosen as a model in this study because of their lower stress levels than female rats (21) since stressful conditions can disrupt blood glucose measurements and trigger potential data bias (22). The health of the rats was assured during the acclimatisation period by administering anthelmintics (Combantrin®, 1 ml/rat) and antibiotics (Amoxsan®, 40 mg/kg BW). The body weight range for the rats was 200-250 grammes. The rats were fed according to laboratory standards (NOVA) and given unlimited water (ad libitum) every day. The rat cage is made of H-Temp polysulfone cage body with a shelf made of stainless steel containing two rats per box. Rat cages were given a base in the form of bedding and were replaced according to the conditions. The room was set at a temperature of 22 ± 3 ºC, with relative humidity 30–70%, and 12 hours of light and of darkness.

The hypoglycaemic activity test was conducted at the Laboratory Animal Management Unit of IPB. This study employed OGTT by injecting larval hydrolysate and other control substances into rats using an oral gavage needle. There were seven treatment groups in this test: the negative control group (water + water), the positive control group (water + glucose), the treatment group (protein hydrolysate doses of 100, 200, 300, 400 mg/kg BW + glucose), and the comparison group (acarbose 4.5 mg/kg BW + glucose). Three rats were used in each treatment group; this number was considered as the minimum sample size based on the results of sample size calculations from the resource equation method formula (23) as follows:

$$n_{min} = (10/k)+1$$

where:
n_{min} = n sample minimum
k = number of treatment groups

The initial glucose level of the rats was measured after 10 minutes of administration of the sample solutions, followed by the administration of 90% glucose solution. Furthermore, blood glucose levels were measured at 30, 60, 90, and 120 minutes (24).

Data analysis

This study used a completely randomised design with four different hydrolysate doses of 100, 200, 300, and 400 mg/kg BW. The data were analysed using ANOVA, and if there were effects from the treatment, the data were continued with Duncan's multiple range test. Statistical analysis of the area under the curve and delta of decreased blood glucose level was performed using the method in the study of Wresdiyati et al. (25).

RESULTS

Insulinotropic amino acid identification using LC-MS/MS

A total of 18 free amino acids were obtained from identification using MassLynx V.4.1 software. Amino acids were then selected based on the insulinotropic type. Selected insulinotropic amino acids include leucine, arginine, lysine, phenylalanine, proline, histidine, and ornithine. Furthermore, the selected amino acid data from the three hydrolysates were compared to determine the hydrolysate with the highest levels and types of insulinotropic amino acids. The selected protein hydrolysate was a sample incubated for 7 hours (H7). The results of insulinotropic amino acids obtained from three BSF larval protein hydrolysate samples are shown in Figure 1.

α-Glucosidase inhibitory assay

H7 underwent further in vitro testing to assess its inhibitory activity on α-glucosidase, compared to a commercially available medicine, acarbose, as the control. Table I shows that H7 exhibited weak inhibition, precisely 3.79% at 1000 µg/g, declining at higher concentrations. The percentage inhibition falls outside the range required to determine the IC50 as it did not reach the lower and upper bounds of 50%. Conversely, as a differential control, acarbose demonstrated near 50% inhibition, precisely 60.33% at 0.5 µg/g, with an IC50 calculated at 0.336 µg/g.

Hypoglycaemic activity using oral glucose tolerance test

As shown in Figure 2, the positive treatment group rats'

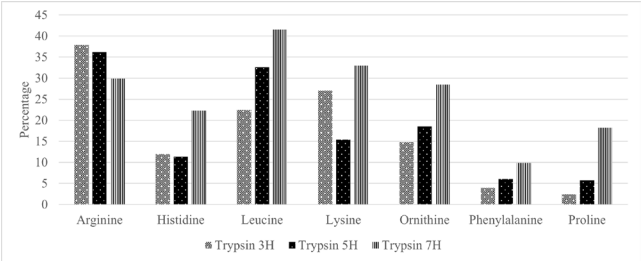

Figure 1: The insulinotropic amino acid levels from BSF larval protein hydrolysate incubated with trypsin for 3, 5, and 7 hours. H = hour of incubation. The highest results for the type and levels of insulinotropic amino acids were shown in Trypsin 7H, followed by 5H, then 3H.

Table I: Result of α- glucosidase inhibitory assay (IC-50) of H7 and acarbose sample

Concentration (µg/mL)	% Inhibition	IC-50 (µg/mL)
H7 Sample		N/A
1000	3.793	
2000	-29.542	
4000	-27.556	
6000	-20.794	
8000	-16.384	
Acarbose		0.336
0.1	21.467	
0.5	60.333	
1	81.298	
5	90.466	
10	96.872	

H7 = larval protein hydrolysate incubated for 7 hours. N/A = not available.

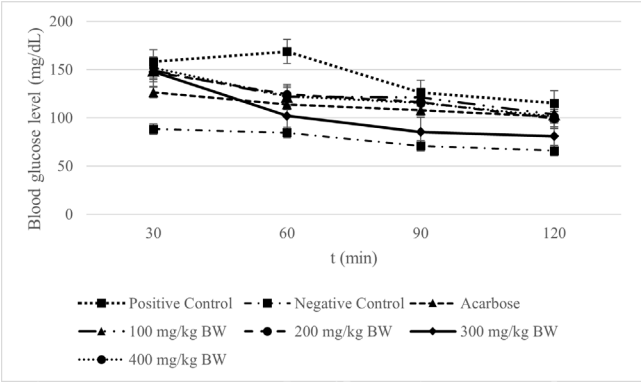

Figure 2: The oral glucose tolerance test result of 7 treatment groups. The blood glucose levels were measured 30 minutes after being given treatment. All hydrolysate-given and acarbose treatment groups experienced a decrease in blood glucose levels after 60 minutes. Only the 300 mg/kg BW dose outperformed the acarbose group in decreasing blood glucose levels. All treatment groups, including the positive control group, showed decreased blood glucose levels after 90 minutes.

blood glucose levels increased at minute 60, whereas the other groups' levels decreased. Furthermore, the negative control treatment group was appointed as the lowest hypoglycaemic point for other hydrolysate treatments. All treatments of larval protein hydrolysate at doses of 100, 200, 300, and 400 mg/kg BW showed a hypoglycaemic effect at minute 60, but only a dose of 300 mg/kg BW provided the best hypoglycaemic effect. This result was supported by the statistical analysis results of the area under the curve presented in Table II. Larval protein hydrolysate at 300 mg/kg BW had the smallest significant area ($P<0.05$) compared with other hydrolysate doses.

The statistical analysis results of delta calculations of blood glucose levels during the observation time of 30–120 minutes for each treatment group are presented in Table III. Based on Table III, it can be seen that larval protein hydrolysate at a dose of 300 mg/kg BW had the highest reduction delta. However, the delta of decreasing blood glucose levels at a dose of 300 mg/kg BW was not significantly different from the doses of 200 and 400 mg/kg BW, and those two doses showed no significant difference with delta-decreased blood glucose levels of the positive control group. The 300-dose treatment group showed a delta of decreased blood glucose levels significantly different from the positive control.

Table II: Area under the curve of rats' blood glucose levels from each treatment

Group	AUC (mg/dL)
Positive control	215.83±3.66^{e}
Negative control	135.90±6.94^{a}
Acarbose	167.83±7.00^{bc}
H 100 mg/kg BW	184.83±3.79^{d}
H 200 mg/kg BW	182.00±5.41^{cd}
H 300 mg/kg BW	155.17±16.26^{b}
H 400 mg/kg BW	187.92±7.08^{d}

$^{a-e}$ means without a common superscript differ ($P < 0.05$). AUC = area under the curve. H = larval protein hydrolysate sample.

Table III: Delta of decreased blood glucose level from each treatment on rats

Group	ΔDBGL (mg/dL)
Positive control	42.67±16.94^{bc}
Negative control	4.33±4.71^{a}
Acarbose	25.33±2.05^{b}
H 100 mg/kg BW	45.33±10.50^{bc}
H 200 mg/kg BW	48.67±7.41^{cd}
H 300 mg/kg BW	66.67±7.72^{d}
H 400 mg/kg BW	50.67±7.13^{cd}

$^{a-e}$ means without a common superscript differ ($P < 0.05$). ΔDBGL = decreased blood glucose level delta. H = larval protein hydrolysate sample.

DISCUSSION

Diabetes mellitus (DM) is a metabolic syndrome characterised by hyperglycaemia. Hyperglycaemia is formed due to disturbances in glucose regulation, such as decreased insulin secretion, increased glucose production, and decreased use of glucose in the body (26). Conditions of chronic hyperglycaemia can induce increased production of free radicals through oxidative stress reactions. This reaction produces a variety of harmful compounds, including advanced glycation

end-products and lipid peroxidation; these compounds play an active role in causing endothelial cell injury/dysfunction (27). If endothelial function is disturbed, vascularisation throughout the body is also disturbed, which can trigger various complications of deadly degenerative diseases (28). Therefore, to suppress the harmful effects caused by hyperglycaemia, there is a solution in the form of a unique strategy that reduces high blood glucose levels through cellular mechanisms. This strategy utilises the unique ability of free amino acids derived from the hydrolysis of BSF larvae. Trypsin enzymes catalyse hydrolysis by separating the sizes and components into free amino acids with bioactive properties (29).

Bioactive amino acids are essential antioxidants for improving the body's physiological functions, especially in reaching homeostasis points (30). Amino acids classified as antioxidants can also fall under the category of insulinotropic compounds, which can suppress insulin synthesis through the sirtuin-1 (SIRT1) pathway within mitochondrial organelles. This significance arises from the pivotal role of SIRT1 in mitochondrial metabolism and the regulation of insulin secretion, adipogenesis, and myogenesis. Furthermore, SIRT1 is critical for modulating glucose metabolism in the liver and pancreas, thereby preventing hyperglycaemia (31). Next, antioxidant amino acids are divided into two groups based on their structure: hydrophobic and aromatic. The hydrophobic group has inhibitory pathways for lipid peroxidation, metal ionisation, and electron transfer processes. Furthermore, aromatic amino acids can stabilise DPPH-type free radicals through the proton donor pathway, facilitating the termination of propagating free radical reactions (32).

The amino acid antioxidant effect of H7 was challenged in vitro using the α-glucosidase inhibitory assay, and the results were unsatisfactory. Zheng et al. (33) reported that α-glucosidase is an enzyme that regulates hyperglycaemia. This enzyme is located on the membrane of the small intestinal epithelial tissue and breaks down oligosaccharides into glucose (34). Oligosaccharides are digested by salivary α-amylase followed by pancreatic α-amylase. The hydrolysis product of the digestion of α-amylase is absorbed on the surface of the brush border epithelial cells of the small intestine by α-glucosidase. Inhibition of α-glucosidase slows starch digestion and suppresses postprandial hyperglycaemia (26). One of the commercially available α-glucosidase inhibitors is acarbose. $C_{25}H_{43}NO_{18}$ is the empirical chemical formula of acarbose, a competitive brush border inhibitor of α-glucosidase (35). Therefore, acarbose was used as a differential control in this assay. The reason for the very weak inhibitory activity of H7 is that the active component of the amino acid does not match the enzyme substrate, so it does not produce a complex bond with the enzyme-substrate, indicating that H7 does not work in the α-glucosidase inhibitory pathway in regulating blood glucose levels. Although H7 has no effect on lowering blood glucose levels based on result of in vitro testing on the nutrient absorption pathway, e.g., the intestine. The hypoglycaemic efficacy using in vivo test is still needed to validate the alleged efficacy of amino acids in regulating homeostasis. Considering that there are many metabolic pathways involved in the biochemical mechanisms, a single system cannot represent in making decisions to determine the efficacy of drug candidates. Therefore, H7 was continued to the OGTT using an animal model of hyperglycaemia.

The OGTT measures blood glucose levels in an animal model of hyperglycaemia. In this case, hyperglycaemia is defined as the result of disturbed fasting blood glucose levels, which have a value of >126 mg/dl (35) but typically can decrease over time without any intervention due to homeostasis (36). A positive control was employed to resemble this typical condition. Therefore, to determine the hypoglycaemic activity of the H7 graded dose treatment group, a lower and earlier reduction in blood glucose levels is required compared with the positive control treatment group.

All treatment groups of H7 showed a blood glucose-lowering effect, confirmed by analysis of the area under the curve and delta of blood glucose reduction. Based on the calculation of the area under the curve, a dose of 300 has an area under the curve equal to that of the comparison group, acarbose, which was noted by superscripts in the same column, which showed that both are not significantly different. In addition, even though doses of 200 and 400 have the same effect as doses of 300 in reducing blood glucose levels statistically, this ability still resembles the positive control treatment group, so the effect caused by doses of 200 and 400 has no significant effect. Thus, delta calculations of blood glucose levels also show that larval hydrolysate at 300 mg/kg BW is the dose with the most optimum hypoglycaemic effect. There is a reason for the phenomenon of a double dose of 400 hydrolysate that resembles a 200 dose. This result could be affected by hyper-aminoacidemia (37), which can induce endocrine feedback by amino acids to stimulate glucagon secretion acutely, resulting in increased stimulation of blood glucose release (38).

Many amino acid metabolism pathways predict hypoglycaemic effects in OGTT results. The amino acid histidine has an imidazole ring structure that acts as a free radical scavenger, and this ability is indispensable for DM therapy (17). Lysine is an insulin-stimulating amino acid that acts through the plasma membrane depolarisation pathway of the cationic uptake system and increases energy with the tricarboxylic acid (TCA) cycle (39). In contrast, proline acts via the sodium co-transport system and increases energy (40). The amino acid in BSF larval protein hydrolysate, which is excellent for blood glucose regulation, is leucine. According to expert reports, leucine is vital in many insulin stimulation

pathways (41). The pathway of insulin binding by leucine activates glucokinase, thereby increasing energy via glycolysis. Leucine can also be converted into α-ketoisocaproate, inhibiting KATP channel activity and increasing extracellular Ca^{2+} uptake. Calcium intake can increase insulin-producing cells (42). The amino acids that are the next mainstay are tryptophan and ornithine. The two amino acids bind to the dipeptidyl peptidase-IV (DPP-IV) enzyme to extend the duration of the hormone incretin. The incretin hormone, which consists of glucose-dependent insulinotropic peptides (GIPs) and glucagon-like peptide-1 (GLP-1), can stimulate insulin production in the pancreas. The amino acids responsible for insulin synthesis through the incretin pathway are arginine, phenylalanine (43), and ornithine. The pathway initiated by ornithine involves Ca^{2+} intake in intestinal L and K cells to increase incretin synthesis (44).

There are limitations to this study. First, the small sample size in this study can lead to potential bias; including a larger sample size in a future study can provide more statistical power. Second, there is uncertainty regarding the type of pathway that decreases blood glucose from the BSF larval protein hydrolysate. Although the in vitro approach does not provide good results regarding the inhibition of α-glycosidase enzymes, the in vivo approach showed a reduction of blood glucose in animal models. Thus, there is a presumption that insulinotropic amino acids have a pathway to reduce blood glucose, which differs from other compounds. The insulinotropic amino acids can balance blood glucose levels through their ability to inhibit the enzyme dipeptidyl peptidase 4. Therefore, there is a need to confirm this in a subsequent study.

CONCLUSION

The best hydrolysate obtained from this study was the sample incubated for 7 hours due to its highest insulinotropic amino acid levels and types compared with the other two hydrolysates. The H7 hydrolysate can exert a hypoglycaemic effect via the insulinotropic pathway and not inhibit the α-glucosidase enzyme, with its optimum dose of 300 mg/kg BW.

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