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Research article

Immunomodulatory potential of protein hydrolysate by-products derived from skipjack (*Katsuwonus pelamis* (Linnaeus, 1758)) against nicotinamide-streptozotocin induced diabetic rat

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Abstract

Type 2 diabetes mellitus (T2DM) is determined by glucose intolerance and low-grade chronic inflammation that might be partially controlled using nutritional interventions. Skipjack tuna (Katsuwonus pelamis) by-products are processed by enzyme hydrolysis, providing many essential elements with health-promoting benefits. Herein, the study aimed to investigate the effect of dietary supplementation of fish protein hydrolysate (FPH) derived from skipjack tuna on diabetes rat models. Twenty-five male Sprague-Dawley rats were separated into five groups: (1) normal group, (2) DM (diabetes mellitus) group, (3) DM + Imunos 0.8 g/kg BW (positive control) group, (4) DM + FPH 0.8 g/kg BW, and (5) DM + FPH 1.6 g/kg BW. The DM groups consecutively gave a high-fat diet (HFD) for twelve weeks. Nicotinamide (NA) (120 mg/kg BW) and streptozotocin (60 mg/kg BW) were then injected to induce diabetic animal models. The treatment was given orally each day for two weeks. Fasting blood glucose, insulin, homeostasis model assessment of insulin resistance (HOMA-IR), HOMA-β, GLUT4, and malondialdehyde (MDA) were measured. Our result suggested that FPH administration increased the body weight of DM rats significantly ($p<0.05$). FPH also reduced the fasting blood glucose, HOMA-IR, HOMA-β, improved insulin and GLUT4 levels, and decreased MDA significantly ($p<0.05$) than DM groups. Our finding suggests FPH derived from skipjack tuna ameliorates DM progression by controlling glucose homeostasis and oxidative stress. Biopeptides from FPH might be a promising candidate as food nutraceuticals that can be involved in DM management.

Keywords: Diabetes mellitus, Fish protein hydrolysate, Insulin, Malondialdehyde, Skipjack tuna

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease with elevated blood glucose levels and affected kidneys, which produce large amounts of urine due to impaired insulin production or action. (Suryasa et al., 2021) Changes in lifestyle and unhealthy diet are increasingly triggering DM and also contribute to a high prevalence of obesity. Obesity is the main risk factor in the development of type 2 diabetes mellitus (T2DM), where more than 85% prevalence of DM is T2DM (Forouhi and Wareham, 2019). The International Diabetes Federation (IDF) estimated that 643 million people will have diabetes in 2030 (11.3% of the world population) and will continue to increase until 783 million in 2045 (IDF, 2021).

Obesity causes inflammation, contributing to insulin resistance, tissue damage, and further complications. Insulin resistance induces inactivation of GLUT4, leading to a decrease in glucose absorption by cells, thus increasing the glucose level in the bloodstream (McNay and Pearson-Leary, 2020). Insulin resistance causes pancreatic β-cells dysfunction and changes the metabolism system to decrease insulin production. Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) is used to assess the level of insulin resistance, and HOMA-β is used to assess the function of pancreatic β-cells in producing insulin (Cao et al., 2024). The reduced ability of β-cells to respond to glucose levels is also caused by calcium imbalance in β-cells, which is caused by glucose metabolism dysfunction. Calcium is essential for β-cells in insulin release (Klec et al., 2019). DM is also associated with increased production of reactive oxygen species (ROS) due to increased glucose levels, resulting in oxidative stress development. Malondialdehyde (MDA) is an end marker of oxidative stress that is derived from lipid peroxidation. High MDA levels indicate oxidative damage contributing to cellular dysfunction, including pancreatic β-cells damage (Mukai et al., 2022).

Currently, treatment for DM is generally carried out through diet adjustments, lifestyle improvements, and medical drugs aimed at regulating blood sugar levels. However, some medications can cause long-term side effects, including gastritis, abdominal pain, bloating, diarrhea, and nausea (Chaturvedi et al., 2018). Developing alternative medication for DM that is safer and more effective is important. Recently, proteins and bioactive peptides have been widely used as natural medications for various diseases. Marine fish is a natural resource that provides high protein and peptides.

Skipjack tuna (Katsuwonus pelamis) is often found in tropical and subtropical waters and is known to have a high protein content with a beneficial essential amino acid profile. Skipjack tuna ranks third globally in the most harvested marine species due to its significant commercial potential. Over 55% of Skipjack tuna is the main raw material for canned fish products. The high demand for skipjack tuna also resulted in high by-products or waste products, including heads, tails, bones, skin, fins, and innards. These by-products are usually used to produce fish oil, fish meal, pet food, or simply become waste products, resulting in the waste of biological resources and causing severe environmental problems (Shi et al., 2024). The fish protein hydrolysate (FPH) procedure can be used to process protein from skipjack tuna by-products. FPH enzymatically breaks down the protein into bioactive peptides (BP) with anti-inflammatory, antidiabetic, and antioxidant activity. The FPH produces bioactive peptides that can inhibit critical enzymes in glucose metabolism, such as α-glucosidase and dipeptidyl peptidase-IV (DPP-IV), which play an important role in the regulation of blood sugar levels by promoting insulin signalling through the AMP-activated protein kinase signaling pathway (AMPK). Additionally, through this AMPK pathway, BP can improve glucose control by increasing glucose absorption and utilization, insulin sensitivity, and protecting β-cells from oxidative stress and damage (Shekoohi et al., 2024).

Considering the importance of effective management of DM and avoiding long-term side effects from medication, FPH from skipjack tuna by-products can

be considered to be used as an alternative treatment for DM. However, the study on FPH from skipjack tuna for DM is still limited. This study aims to produce FPH from skipjack tuna by-product and evaluate the BP activity on important markers of DM, including blood glucose levels, insulin, GLUT4, and malondialdehyde (MDA) in diabetic rats.

MATERIALS AND METHODS

Samples preparation

The sample is a by-product of skipjack tuna obtained from Cilacap Regency, Central Java, Indonesia. Samples of skipjack tuna that had been deboned and cut were then processed using a protein hydrolysis procedure referring to the method of Prasetyo et al. (2021), with slight modifications. A total of 100 g of sample was put into distilled water (1:3) and added 5% papain enzyme (0.0835 ± 0.0009 U/mL). The mixture was maintained at pH 6.4 during hydrolysis by adjusting the pH using 0.1 M NaOH or 0.1 M CH_3COOH. The hydrolysis process was carried out at 61°C for 230 minutes. Then, the enzyme was inactivated at 80°C for 30 minutes. The hydrolysis results were filtered using Whatman paper no. 43 and then dried using a spray dryer (Buchi mini spray dryer B-290, manual procedure) with an inlet temperature of 140°C and an outlet of around 95°C. The hydrolysis resulting powder was then stored at -20°C for further analysis.

Animal and Experimental Design

Twenty-five male Sprague-Dawley rats (8±2 weeks old, 170±10 g of body weight) were obtained from the Inter-University Animal Center, Gadjah Mada University. Rats were acclimatized for one week, housed in standard cages with free access to food and water individually. Rats (n=20) were fed a high-fat diet (HFD) for 3 months using HFD32, which contains 32% crude fat and 60% calories by Dr. Osamu Ezaki with modified gross energy (Fat kcal%). The HFD32 formulation can be seen in Table 1.

Table 1 HFD32 ingredients

Ingredients	Percentage
Milk casein	20.500
Egg white	20.500
L-cystine	0.368
Powdered beef tallow (including 80% of beef tallow)	13.290
Safflower oil (high oleic acid)	16.750
Crystalline Cellulose	4.600
Maltodextrin	6.900
Lactose	5.800
Sucrose	5.655
AIN93 vitamin mix	1.155
AIN93G mineral mix*	4.180
Choline bitartrate	0.300
Tertiary butylhydroquinone	0.002
Total	100.000

At the end of the HFD, the blood glucose levels were measured as initial blood glucose. Rats were then injected with several doses of nicotinamide (NA) (120 mg/kg BW) and then injected with a low dose of streptozotocin (STZ) (60 mg/kg BW) one hour later. STZ stock solution was prepared by dissolving 160 mg STZ in 16 mL 0.1 M citrate buffer at pH 4.5. The fasting blood glucose levels from the tail vein were measured a week later. Fasting blood glucose levels ≥200 mg/dL were considered diabetes. The rats were then divided into five groups, namely: (1) normal group, (2) DM (diabetes mellitus) group, (3) DM + Imunos 0.8 g/kg BW (positive control) group, (4) DM + FPH 0.8 g/kg BW, and (5) DM + FPH 1.6 g/kg BW. The treatment was given orally every day for two weeks. The body weight and food intake of rats were measured weekly during the experiment.

Rats were fasted for 12 h, and fasting blood glucose levels were measured as final blood glucose levels. Rats were injected intramuscularly with Ket-A-Xyl (AgorVet, Peru) according to the dose recommended by the product manufacturer and then dissected. The blood was drawn through the cardiac aorta and placed in a gel clot vacutainer tube. Serum was obtained by centrifuging at 3000 rpm for 10 minutes at 10°C. The serum was then kept at -20oC for further analysis.

The Bioethics Committee has approved this research protocol relating to animal welfare for Medical/Health Research of The Faculty of Medicine, Sultan Agung Islamic University, with approval number 202/V/2023. This procedure also complies with the principles of laboratory animal care by the National Institutes of Health National Research Council (US) Committee for Update of Guidelines for the Care and Use of Laboratory Animals.

Measurement of insulin, GLUT4, and MDA levels

The levels of insulin, GLUT4, and MDA in the serum were measured using the enzyme-linked immunosorbent assay (ELISA) method with the ELISA kit for insulin ELR-Insulin-1 (RayBiotech, USA). In contrast, the ELISA kit for GLUT4 was E-EL-R0430 by Elabscience (Texas, USA), and the ELISA kit for MDA was E-EL-0060 by Elabscience (Texas, USA). The ELISA procedures followed the manufacturer's procedures.

Measurement of HOMA-IR and HOMA-β

HOMA-IR values between 0.5 - 1.4 are considered normal;> 1.9 indicates early IR, and ≥2.9 indicates IR (Jiménez-Maldonado et al., 2020). HOMA-IR measurements are determined using the formula:

$$\text{HOMA-IR} = \frac{\text{Fasting plasma glucose}(\frac{mg}{dl}) \times \text{fasting insulin level}(\mu\frac{U}{ml})}{405}$$

HOMA-β values >110% were considered normal, and <80% were considered low (Jiménez-Maldonado et al., 2020). HOMA-β measurements can be calculated using the formula:

$$\text{HOMA-}\beta = \frac{(360 \times \text{fasting insulin level}(\mu\frac{U}{ml})}{\text{Fasting plasma glucose}\left(\frac{mg}{dl}\right) - 63}$$

Statistical Analysis

The data were analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) as a post hoc test. The different superscript letter indicates a significant difference among groups ($p < 0.05$), while the same superscript letter indicates no significant difference among groups ($p > 0.05$).

RESULTS

FPH controlling the body weight and blood glucose levels in diabetic rat model

Based on the data analysis, the body weight of rats before treatment did not significantly differ (Figure 1), with an average body weight of 222.67 g. After STZ induction, the body weight of the DM group without treatment decreased significantly compared with other groups. This result suggested that weight loss can be a symptom of diabetes due to dysfunction in glucose and insulin regulation.

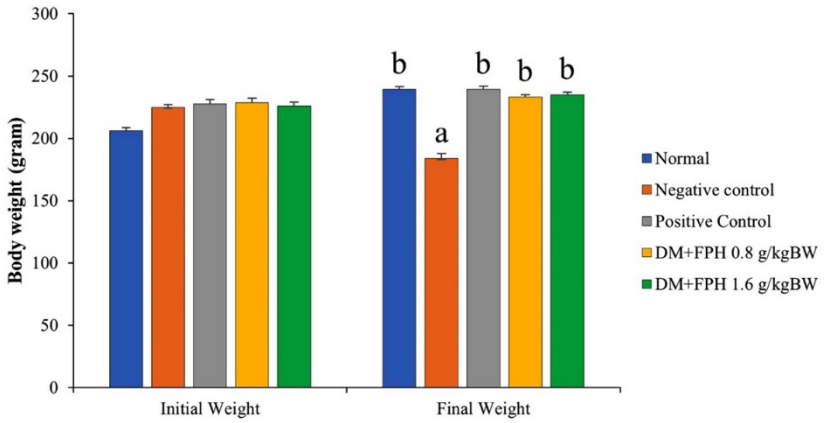

Figure 1 The body weight changes at the initial and final treatment. Normal, non-diabetic rats; Negative control rats, DM rats without treatment; Positive control rats, DM rats treated with drug control Imunos 0.8 g/kg BW; DM+FPH 0.8 g/kg BW, DM rats treated with fish protein hydrolysate dosage 0.8 g/kg BW; DM+FPH 1.6 g/kg BW, DM rats treated with fish protein hydrolysate dosage 1.6 g/kg BW. The different letters above the bar chart indicate a significant difference ($p<0.05$) among groups based on DMRT

The initial blood glucose of the animal model was in the range of 66-69 mg/dL. After STZ injection, the DM rats reached blood glucose up to 270 mg/dL and showed a significant difference compared with normal rats (Figure 2). The final blood glucose levels showed that treatment using FPH from skipjack tuna significantly decreased the blood glucose levels compared with a negative control group. The administration of FPH also lowers the blood glucose levels in DM rats, which is close to the normal group.

FPH improve the HOMA-IR and HOMA-β in diabetic rat model

Based on Figure 3, the negative control group has the highest value of HOMA-IR and the lowest value of HOMA-β, which means that the negative control group was in the IR state with a decrease in β-cells to produce insulin. Treatment using FPH from skipjack tuna by-product can increase the HOMA-β and ameliorate the HOMA-IR significantly compared with the negative control group. Improvement on HOMA-IR and HOMA-β can be used to consider that treatment using FPH from skipjack tuna by-product repaired the pancreatic β-cells to produce and increase insulin sensitivity.

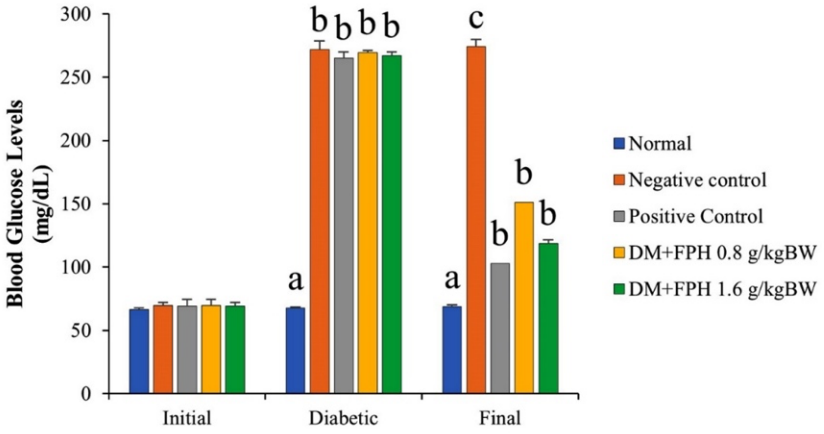

Figure 2 The blood glucose levels at initial, diabetic condition, and final treatment. Normal, non-diabetic rats; Negative control rats, DM rats without treatment; Positive control rats, DM rats treated with drug control Imunos 0.8 g/kg BW; DM+FPH 0.8 g/kg BW, DM rats treated with fish protein hydrolysate dosage 0.8 g/kg BW; DM+FPH 1.6 g/kg BW, DM rats treated with fish protein hydrolysate dosage 1.6 g/kg BW. The different letters above the bar chart indicate a significant difference ($p<0.05$) among groups based on DMRT

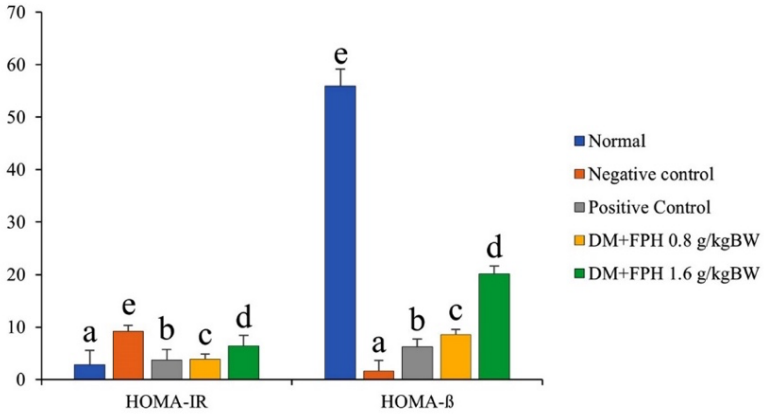

Figure 3 HOMA-IR and HOMA-β value between normal and diabetic rats. Normal, non-diabetic rats; Negative control rats, DM rats without treatment; Positive control rats, DM rats treated with drug control Imunos 0.8 g/kg BW; DM+FPH 0.8 g/kg BW, DM rats treated with fish protein hydrolysate dosage 0.8 g/kg BW; DM+FPH 1.6 g/kg BW, DM rats treated with fish protein hydrolysate dosage 1.6 g/kg BW. The different letters above the bar chart indicate a significant difference ($p<0.05$) among groups based on DMRT

FPH restore the levels of insulin in a diabetic rat model

Furthermore, based on the insulin levels analysis showed that the DM rat group has lower insulin levels compared with the FPH treatment groups (Figure 4). Insulin levels might decrease in diabetic conditions due to impaired blood glucose levels. FPH administration showed improved blood glucose levels (Figure 2), followed by increased insulin levels in the serum significantly compared with the DM rat group.

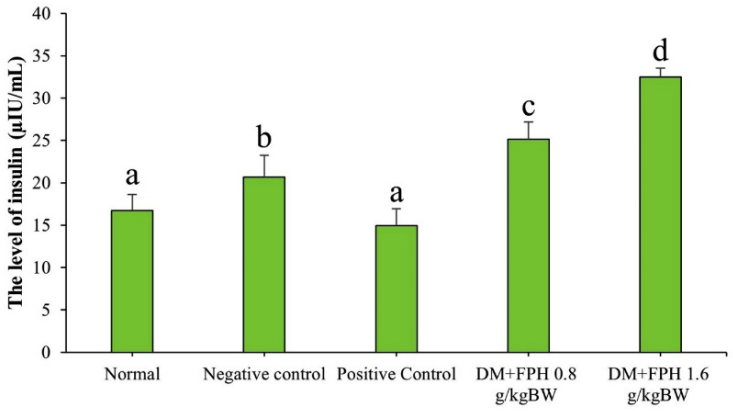

Figure 4 The insulin levels in the serum in normal and diabetic rats after treatment using FPH from skipjack tuna by-product. Normal, non-diabetic rats; Negative control rats, DM rats without treatment; Positive control rats, DM rats treated with drug control Imunos 0.8 g/kg BW; DM+FPH 0.8 g/kg BW, DM rats treated with fish protein hydrolysate dosage 0.8 g/kg BW; DM+FPH 1.6 g/kg BW, DM rats treated with fish protein hydrolysate dosage 1.6 g/kg BW. The different letters above the bar chart indicate a significant difference ($p<0.05$) among groups based on DMRT

FPH ameliorates the GLUT4 levels in the diabetic rat model

Data analysis on the level of GLUT4 showed that DM rats without treatment (negative control group) have the lowest level of GLUT4, while normal has the highest level of GLUT4 in the serum (Figure 5). FPH treatment showed a significant increase in GLUT4 compared with the negative control group. Furthermore, FPH also gives better results in improving GLUT4 levels compared with drug control groups. This result suggested that FPH treatment from skipjack tuna by-products implies repairing GLUT4 function in glucose metabolism and transport.

FPH decreased MDA levels in the diabetic rat model

Figure 6 indicates that the DM rat group has the highest level of MDA. HFD fed following STZ-induced diabetic rats causes elevating oxidative stress due to the lipid peroxidation process. This mechanism increased MDA significantly as the end-product of the peroxidation process on saturated fatty acid. FPH administration decreased the MDA level significantly compared with diabetic rats without treatment. FPH, especially at a dosage of 1.6 g/kg BW, showed better results than the drug control group and positively affected the MDA levels decrease, similar to the normal group. FPH from skipjack tuna by-products suggested its ability as an antioxidant, which can be used to improve the oxidative stress stage.

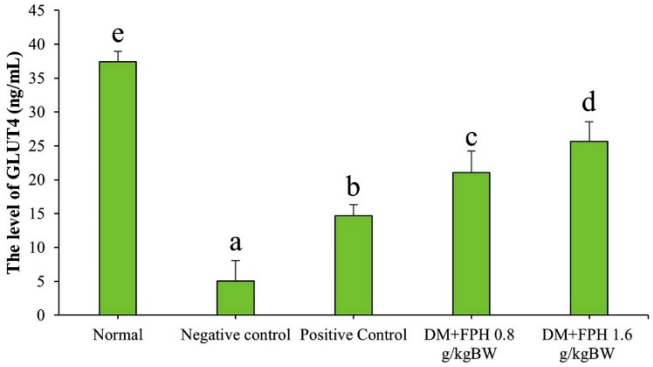

Figure 5 The level of GLUT4 in the serum in normal and diabetic rats after treatments. Normal, non-diabetic rats; Negative control rats, DM rats without treatment; Positive control rats, DM rats treated with drug control Imunos 0.8 g/kg BW; DM+FPH 0.8 g/kg BW, DM rats treated with fish protein hydrolysate dosage 0.8 g/kg BW; DM+FPH 1.6 g/kg BW, DM rats treated with fish protein hydrolysate dosage 1.6 g/kg BW. The different letters above the bar chart indicate a significant difference ($p<0.05$) among groups based on DMRT.

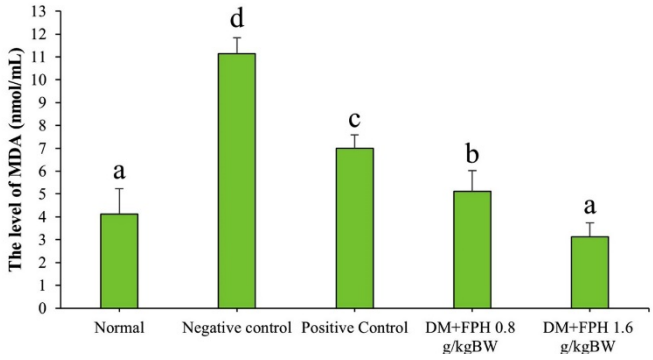

Figure 6 The level of MDA in the serum in normal and diabetic rat groups after being treated using control drug and FPH. Normal, non-diabetic rats; Negative control rats, DM rats without treatment; Positive control rats, DM rats treated with drug control Imunos 0.8 g/kg BW; DM+FPH 0.8 g/kg BW, DM rats treated with fish protein hydrolysate dosage 0.8 g/kg BW; DM+FPH 1.6 g/kg BW, DM rats treated with fish protein hydrolysate dosage 1.6 g/kg BW. The different letters above the bar chart indicate a significant difference ($p<0.05$) among groups based on DMRT.

DISCUSSION

Bioactive peptides derived from food sources are sufficient natural materials to regulate blood glucose, along with additional benefits such as antihypertensive, antioxidant, antimicrobial, and antiproliferative properties. One method that shows promise for producing bioactive peptides with enhanced activity over their precursors is enzyme hydrolysis. FPH contains proteins, peptides, and ω-3 polyunsaturated fatty acids, which might serve as antidiabetic candidates. Fatty acids have been proposed to enhance wound healing and provide protection against sepsis, together with additional complications arising from impaired bodily responses to wounds caused by elevated glucose levels (Pintapagung et al., 2020).

The bioactive compounds from FPH have been well-studied in recent years to manage DM complications (Zhou et al., 2021).

In general, DM in animal models was accompanied by body weight loss. STZ-induced diabetes mellitus is characterized by significant weight loss, which may be attributed to the breakdown of structural proteins contributing to body weight (Chijiokwu et al., 2022). Therefore, an improvement in body weight predicts the positive effect of treatment on DM. Our result demonstrated that FPH administration improves the body weight in diabetic rats. Similar results were displayed with other marine products, such as *Palmaria palmata* and *Trachinotus ovatus* hydrolysate, which increased the body weight of diabetic rats (McLaughlin et al., 2020; Wan et al., 2023). Blood glucose alteration after the initial STZ injection followed by treatment might reflect the progress of DM (Fungfuang et al., 2016). Our result demonstrated that FPH reduced glucose levels in diabetic rats. FPH from skipjack tuna has shown potential in reducing blood glucose levels in diabetic rats. A study on skipjack tuna (*K. pelamis*) viscera protein hydrolysate demonstrated high protein content (81.04%) and essential amino acids (43.13%), suggesting its potential as a source of bioactive peptides (Klomklao and Benjakul, 2017). FPH from skipjack tuna was predicted to have glucose uptake stimulating peptide, DPP-III and IV inhibitor, and alpha-glucosidase inhibitor properties (Riyadi et al., 2023). Another study using Selar fish (*Selar crumenophthalmus*) protein hydrolysate revealed that blood glucose regulation might be increased by GLP-1 expression (Kusuma et al., 2021).

In line with the blood glucose result, there is an improvement in the insulin levels of diabetic rats after treatment with FPH. A previous study reported that protein hydrolysates from salmon and mackerel heads could improve insulin sensitivity (Daskalaki et al., 2023). Interestingly, peptides with 3 to 15 amino acids containing numerous typical residues of QARLFPVIM have been found to play an essential role in antidiabetic activities by inhibiting DPP-IV activity, stimulating GLP-1 secretion, and improving insulin secretion (Harnedy et al., 2018; Harnedy-Rothwell et al., 2020). Another study suggested that oligopeptide from chum salmon could upregulate the GLUT4 expression (Zhu et al., 2017). In the present study, FPH improved insulin levels by controlling blood glucose and GLUT4. An improvement in the insulin signaling pathway may trigger the activation of the PI3K/Akt signaling pathway and GLUT4 translocation, which results in improved blood glucose levels (Wang et al., 2019).

MDA is commonly used to determine the oxidative stress status. Thus, preventing the free radical synthesize might be beneficial to diminish diabetes progression (Gofur et al., 2018; Riyadi et al., 2020). FPH is suggested to promote enzymatic antioxidants, which inhibit lipid peroxidation and protect from acute lung injury (Riyadi et al., 2022). Aromatic and hydrophobic amino acids, including Ala, Val, Tyr, Met and His, could facilitate the interaction between peptides and free radicals by increasing the solubility of the peptide in lipids (Sila and Bougatef, 2016; Wong et al., 2019). For example, His has an imidazole ring that can serve as a lipid peroxyl radical trap, hydrogen, and proton donor (Wu et al., 2018). Met in FPH have a site important for scavenging free radicals and play a pivotal role in biopeptide antioxidative activity. In addition, Tyr acts as a hydrogen donor to diminish free radicals and terminate the chain reaction of peroxidation (Wu et al., 2018; Zhang et al., 2019). Other amino acids, such as Glu, Asp, and Lys, were reported as hydrophilic amino acids with metal chelating and radical scavenging activity (Zheng et al., 2018). Thus, FPH with molecular weights below 3 kDa has been reported to have stronger alpha-amylase and DPP-IV inhibitory and antioxidant activity (Taheri and Bakhshizadeh, 2020; Wan et al., 2023). This finding suggests that skipjack-derived protein hydrolysates could restore glucose homeostasis and mitigate oxidative stress in diabetic conditions.

CONCLUSIONS

In summary, FPH from *K. pelamis* significantly ameliorates body weight, glucose tolerance, and free radicals in DM rats. Bioactive peptides provide an important insight into being involved in DM management. Further studies are required to gain a detailed mechanism of biopeptides from fish hydrolysate, protecting them from DM complications. Moreover, the FPH from K. pelamis might develop as a protein-rich functional food derived from underutilized fish organs to add value and health benefits.

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AUTHOR CONTRIBUTIONS

Conceptualization: PHR, Methodology: PHR, ES, TWA, MFA, SNA, Validation: PHR, Funding acquisition: PHR Writing – original draft: MFA, SNA Writing – review & editing: PHR, ES, TWA, MR. All authors read and approved of the final manuscript.

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