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Evaluation of Immunomodulatory Properties of Fish-Protein Hydrolysate from Skipjack Tuna by-products (*Katsuwonus pelamis*, Linnaeus 1758) in Streptozotocin-Nicotinamide-Induced Diabetic Rats

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ABSTRACT

Fish protein hydrolysate (FPH) is an alternative to managing fish by-products. Protein hydrolysis by proteolytic enzymes breaks down the protein into bioactive peptides (BP). Bioactive has a high-protein content and various beneficial health properties, including antioxidants, immunoregulatory, antibacterial, anti-inflammatory, and other activities. The current study aimed to investigate the anti-diabetic and immunomodulatory activities of FPH from skipjack tuna by-products. Male *Sprague Dawley* rats (n = 25) were equally divided into five groups: healthy group, diabetic mellitus (DM) group, DM + Imunos 0.8 g/ kg BW (drug control group), DM + 0.8 g/kg BW (FPH 1), DM + 1.6 g/kg BW FPH (FPH 2). Diabetic rats were induced by being fed with a high-fat diet (HFD) for 3 months, followed by nicotinamide (NA; 120 mg/kg BW)-streptozotocin (STZ) injection (60 mg/kg BW). The initial and final body weights before and after treatment were measured. The leukocyte and lymphocyte levels were measured using a hematology analyzer. The pro-inflammatory cytokine tumor necrosis factor α (TNFα) level was measured using enzyme-linked immunosorbent assay (ELISA). The result showed that the blood glucose levels after treatment using FPH significantly decreased compared with DM rats. Leukocyte and lymphocyte numbers also decreased significantly after treatment using FPH 1 than in DM rats. The pro-inflammatory cytokine TNFα in the FPH rat groups improved significantly compared with DM rats. These study results suggested that FPH from skipjack tuna by-product administration can be used as anti-diabetic and immunomodulatory candidates.

Keywords: Diabetes mellitus, Fish protein hydrolysate, Inflammation, Skipjack tuna by-product

INTRODUCTION

Skipjack tuna (*Katsuwonus pelamis*) is a pelagic migratory fish belonging to the Scombridae family. Skipjack tuna is found in tropical and subtropical waters and has a high commercial value (Artetxe-Arrate et al., 2021; Shin et al., 2024). The catches of tuna and tuna-like species have increased in recent years, reaching their highest levels in 2018 at over 7.9 million metric tons (FAO, 2020). Tuna is mostly used as raw material for canned tuna products, resulting in a rapid increase in product demand over the past four decades (Kawamoto, 2022). The fish processing industry mostly produces 25-70% by-products or waste in the form of skin, head, fins, tail, bones, and offal (Wang et al., 2022; Abeysinghe et al., 2024). These by-products are usually used in producing pet food, animal feed, fish meal, and fertilizer, or only become waste products, resulting in the waste of biological resources and causing serious environmental problems (Kim et al., 2019; Tacias-Pascacio et al., 2021; Cai et al., 2022). Fish protein hydrolysate (FPH) is an alternate solution to decrease environmental issues that come up caused by fish by-products (Honrado et al., 2024).

Fish Protein Hydrolysate (FPH) is a processed fish waste product in liquid or powder form and consists of bioactive peptides (BP, Daroit and Brandelli, 2021). The enzymatic hydrolysis by proteolytic enzymes helps to hydrolyze the protein into short-chain peptides to produce active peptides (Caruso et al., 2020; Fadimu et al., 2022). Fish Protein Hydrolysate was reported to increase solubility, emulsifying properties, foaming, water-holding capacity, and fat-binding capacity (Dinakarkumar et al., 2022). Moreover, active peptides have many biological functions, such as antihypertensive, antioxidant, immunoregulatory, antibacterial, anti-inflammatory, anti-aging, and other activities (Fadimu et al., 2022; Ye et al., 2022; Ortizo et al., 2023). Bioactive peptides (BP) rarely accumulate in the human body, thus, it has low side effects (Nourmohammadi and Mahoonak, 2018; Akbarian et al., 2022). Meanwhile, the consumption of synthetic drugs has side effects due to high drug residue in the body (Bhardwaj and Misra, 2018).

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Diabetes mellitus, a common metabolic disorder, is linked to notable alterations in the immune system, especially regarding leukocyte and lymphocyte levels. Multiple studies indicate that inflammatory markers, including total leukocyte count, are associated with the onset and advancement of diabetes (Holt et al., 2024). The global prevalence of diabetes and obesity is rising, concerning more than 380 million and 500 million individuals, respectively (Rivero-González et al., 2017). Inflammation is now recognized as a crucial factor in the pathogenesis of both type 1 and type 2 diabetes, leading to a complex interplay between the inflammatory and immune systems (Rohm et al., 2022). Diabetic mellitus (DM) is linked to elevated reactive oxygen species and proinflammatory cytokine levels (Goycheva et al., 2023). Tumor necrosis factor-α (TNF-α) is a significant mediator in the inflammatory process and has essential roles in the development of insulin resistance and the pathogenesis of diabetes (Akash et al., 2018). Bioactive peptides represent a promising therapeutic for DM management, given their ability to affect multiple metabolic pathways and regulate immune responses (Antony and Vijayan, 2021).

Recent studies have suggested that certain peptides found in fish products may possess anti-diabetic properties (Alyari Gavaher et al., 2022) via multiple mechanisms, such as inhibition of the enzyme dipeptidyl peptidase-IV, which plays a role in the stabilization of blood glucose levels (Fakih and Dewi, 2021). Furthermore, FPH is reported to have antioxidant activity and antimicrobial activity in Gram-positive and Gram-negative bacteria (Da Rocha et al., 2018). This current study aimed to elucidate the advantages of FPH from skipjack by-products to maintain blood glucose levels and reduce pro-inflammatory cytokines in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Ethical approval

The animal welfare and experimental procedures were approved by the Animal Ethics Committee of Brawijaya Ethics Committee, Brawijaya University with approval number 181-KEP-UB-2024 and following the principles of laboratory animal care by the National Institute of Health National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals.

Preparation of skipjack tuna by-products protein hydrolysate

The samples in this research were framed and trimmed of skipjack tuna by-products. The samples were obtained from Cilacap Regency, Central Java, Indonesia. The procedure of protein hydrolysis from skipjack tuna followed the procedure from Prasetyo et. al. (2024). 100 g of the sample was added to distilled water with a ratio of 1:3, and then 5% papain enzyme (0.0835 ± 0.0009 U/mL) was added to the mixture. During hydrolysis, the mixture was maintained at pH 6.3 by adding 0.1 M NaOH or 0.1 M CH_3COOH. The temperature of hydrolysis was at 61 °C for 230 minutes, followed by enzyme inactivation at 80^{o}C for 30 minutes. The hydrolysis result was filtered using Whatman paper number 43, then dried using a spray dryer (manual procedure Buchi mini spray dyer B-290, BÜCHI Labortechnik AG, Switzerland) with an inlet temperature of 140 °C and an outlet of ± 95 °C. The hydrolysate powder was then kept at -20 °C for further analysis.

Animals and experimental design

Twenty-five male Sprague-Dawley rats (8±2 weeks old, 170 ± 10 g body weight) were purchased from the Animal Center of Pusat Antar Universitas (PAU), Gadjah Mada University, Indonesia. The animals were housed individually in standard cages and had free access to food and water *ad libitum*. The rats were acclimated for one week before the beginning of the experiment. Rats (n = 20) were administered a modified high-fat diet (HFD32) comprising 32% crude fat, with a caloric contribution from fat sources accounting for 60% of total energy, as formulated by Dr. Osamu Ezaki (National Institute of Health and Nutrition). The HFD32 was given for three months. The HFD32 formulation is presented in Table 1.

Following 3 months of high-fat diet feeding, blood glucose levels were assessed in the rats using blood samples from the tail vein. The rats were subsequently administered multiple doses of nicotinamide (NA, 120 mg/kg BW), followed by several low doses of streptozotocin (STZ, 60 mg/kg BW) one hour after the NA injection (Ghasemi and Jeddi, 2023; Jeong et al., 2024). The STZ stock solution was prepared by dissolving 160 mg of STZ in 16 mL of 0.1 M citrate buffer at pH 4.5. The NA stock solution was prepared by dissolving 100 mg of NA in 10 mL of 0.1 M phosphate buffer at pH 7. Fasting blood glucose levels in rats were assessed one week later through the tail vein. The fasting blood glucose ≥ 200 mg/dL was considered diabetic. The rats were then divided into five groups namely; normal as a normal control group, DM as the diabetic model control group, DM+Imunos (Lapi, Indonesia) 0.8 g/ kg BW (drug control group), DM+FPH1 at a dose of 0.8 g/kg BW, DM+FPH2 at a dose of 1.6 g/kg BW. Fish Protein Hydrolysate dosage determination is determined by dose-response considerations. The rats received the medicines orally daily for two weeks.

Weekly measurements of body weight and feed consumption tracked the experiment. Rats were fasted for twelve hours, and final blood glucose levels were examined. The rats were then dissected using Ketamine-A-Xylazine following the dosage according to the manufacturer's product (Ket-A-Cy, AgorVet, Peru). The blood was collected by cardiac puncture and then placed in a vacutainer ethylene diamine tetra acetic acid (EDTA) tube for leukocyte and lymphocyte measurement using an automatic hematology analyzer (ABX Micros 60, Horiba, Japan). Serum was obtained by collecting the blood in a vacutainer gel clot and then centrifuged at 3000 rpm, 10 ^{o}C for 10 minutes.

Table 1. High-fat diet 32 ingredients for Sprague-Dawley male rats (8 ± 2 weeks old) in 3 months

Formulation	Percent
Milk casein	20.50
Egg white	20.50
L-cystine	0.368
Powdered beef tallow (including 80% of beef fat)	13.29
Safflower oil (high oleic acid)	16.75
Crystalline cellulose	4.60
Maltodextrin	6.90
Lactose	5.80
Sucrose	5.655
AIN93 vitamin mix	1.155
AIN93G mineral mix*	4.18
Choline bitartrate	0.30
Tertiary butyl hydroquinone	0.002
Total	100.000

*A commercial mineral mix for rodents

Measurement of tumor necrosis factor α

The levels of tumor necrosis factor α (TNFα) in the serum were measured using the enzyme-linked immunosorbent assay (ELISA) method using an ELISA kit rat anti-TNFα (ER1393, FineTest, Wuhan, China) and following the manufacturer's procedures.

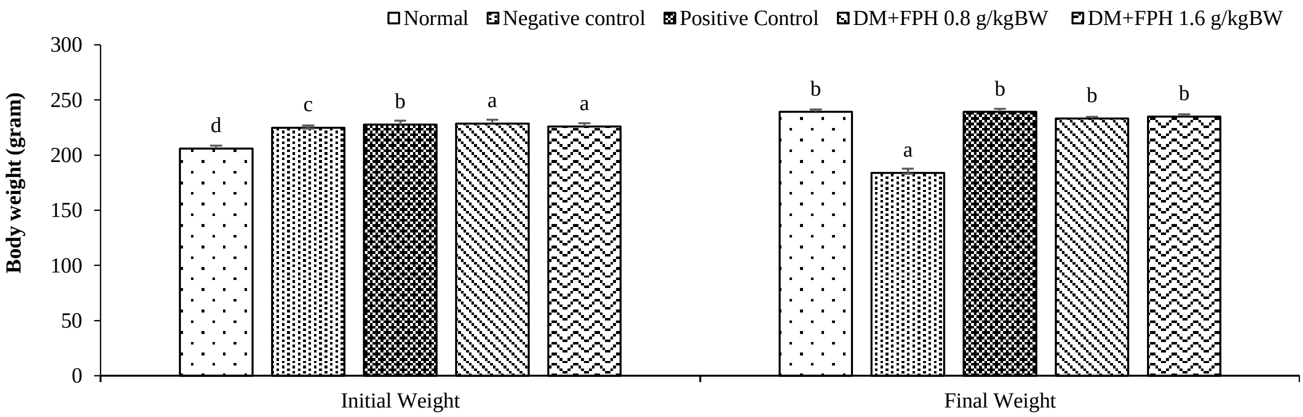
Statistical analysis

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 using Statistical Package for the Social Sciences (SPSS) version 20 (IBM Corp, Armonk, NY). $p < 0.05$ indicated a significant difference between groups.

RESULTS

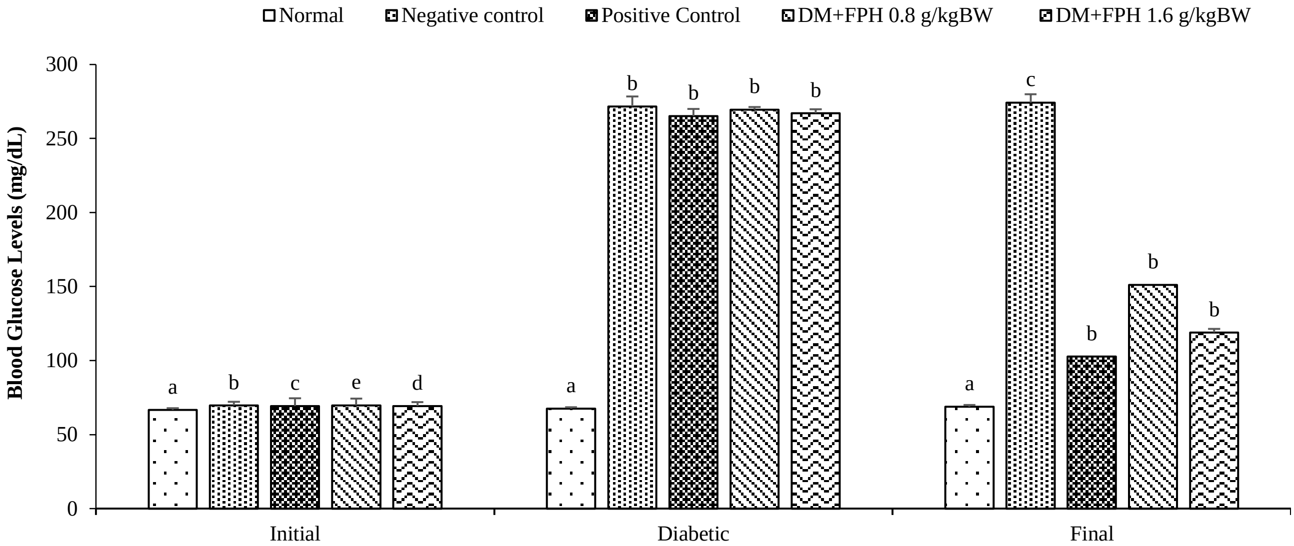
Effects of fish protein hydrolysate on body weight and glucose levels

The body weight of rats at the initial stage showed a significant difference between the groups (Graph 1, $p < 0.05$). The body weight of rats is in the range of 170 ± 10 g. High-fat diet-fed rats demonstrated a significant increase ($p < 0.05$) in body weight than normal groups that fed standard diets ($p < 0.05$). After STZ injection, the diabetic rat groups displayed a significant difference between groups ($p < 0.05$). These results indicated that the HFD fed before STZ induction affected the body weight gain of rats. The normal groups fed a standard chow diet showed a slight body weight increase, while rats fed with HFD32 increased their body weight significantly than the normal groups ($p < 0.05$).



Graph 1. Body weight change of rats at the initial stage and final stage. Normal: Normal control rats; DM: STZ-induced diabetic rats; DM+FPH1: Diabetic rats treated with fish protein hydrolysate dosage 0.8 g/kg BW; DM+FPH2: Diabetic rats treated with fish protein hydrolysate dosage 1.6 g/kg BW. [abcd] Different superscript letters indicated significant differences ($p < 0.05$).

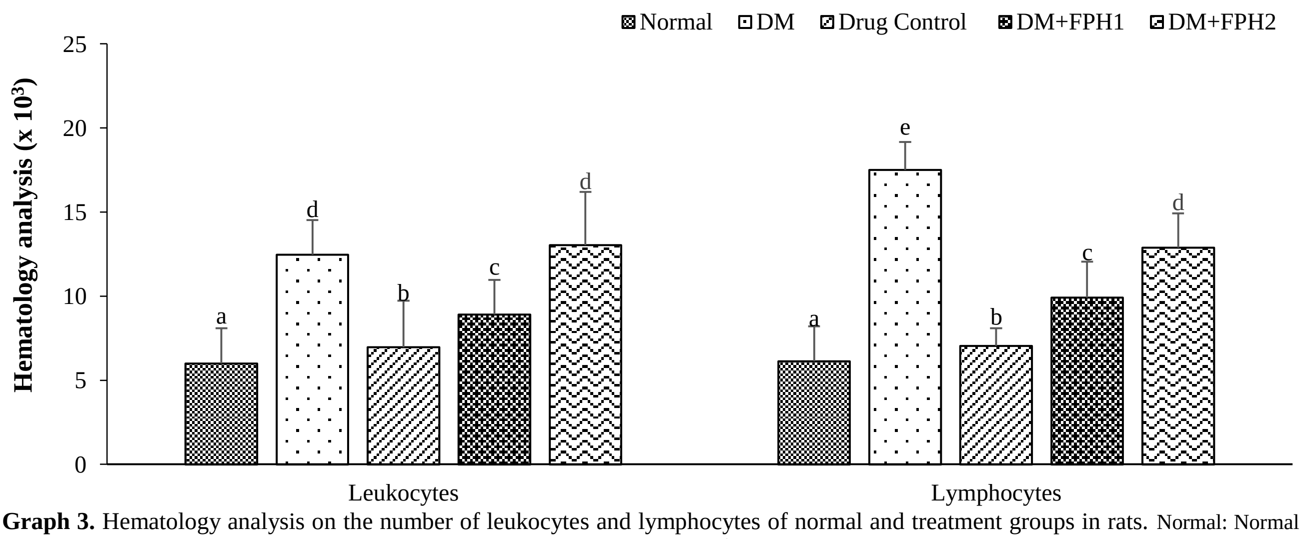
Based on the analysis as seen in Graph 2, the initial glucose levels significantly differ between groups ($p < 0.05$). After the STZ induction, the blood glucose levels in HFD-fed rats increased significantly compared to normal rats ($p < 0.05$). The blood glucose levels after STZ induction reached > 200 mg/dL. After treatment using drug control and FPH, the blood glucose levels decreased significantly compared with the DM rat group ($p < 0.05$). The blood glucose decreased by 43-55% after FPH treatment. Furthermore, decreasing blood glucose levels after treatment were also close to the normal group. This result indicated that FPH could be used to decrease blood glucose under diabetic conditions and has an ability close to drug control.



Graph 2. Blood glucose levels of rats at the initial stage, diabetic stage, and final stage. Normal: Normal control rats; DM: STZ-induced diabetic rats; DM+FPH1: Diabetic rats treated with fish protein hydrolysate dosage 0.8 g/kg BW; DM+FPH2: Diabetic rats treated with fish protein hydrolysate dosage 1.6 g/kg BW. [abcde] Different superscript letters indicated significant differences ($p < 0.05$).

Effects of fish protein hydrolysate on leukocyte and lymphocyte levels

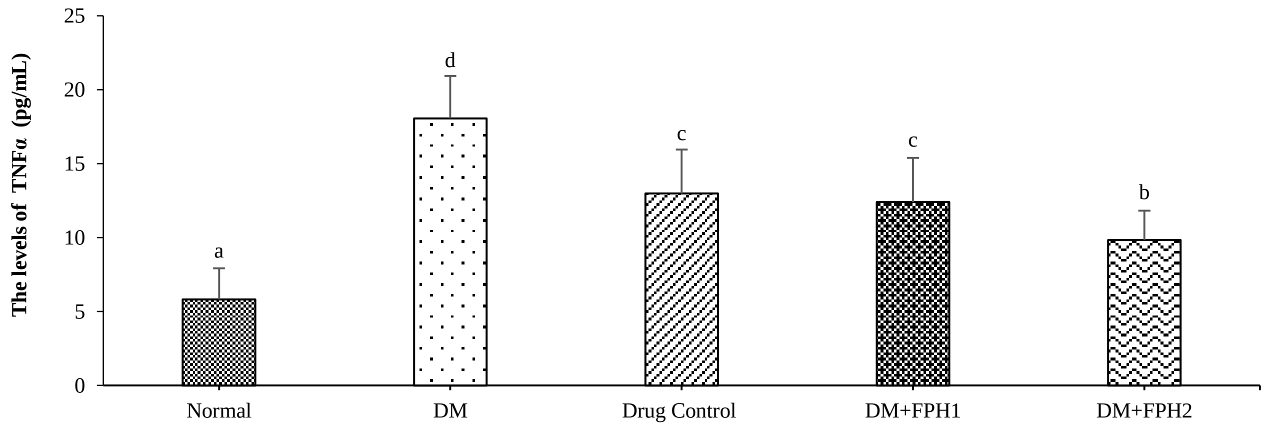
The hematology analysis on leukocytes and lymphocytes showed significant differences between groups (Graph 3, $p < 0.05$). The normal rats group showed the lowest number of both leukocytes and lymphocytes, while the DM rats group had the highest number of both leukocytes and lymphocytes. This result indicated that in diabetic conditions, the number of leukocytes and lymphocytes increased significantly ($p < 0.05$) compared to normal conditions. Furthermore, after treatment using drug control and FPH, the number of leukocytes and lymphocytes significantly decreased ($p < 0.05$). The drug control has a lower number of leukocytes and lymphocytes compared with FPH treatment, however, the treatment using FPH, especially at a dose of 0.8 g/kg BW, showed a significant difference compared with the DM rat group ($p < 0.05$).



Graph 3. Hematology analysis on the number of leukocytes and lymphocytes of normal and treatment groups in rats. Normal: Normal control rats; DM: STZ-induced diabetic rats; DM+FPH1: Diabetic rats treated with fish protein hydrolysate dosage 0.8 g/kg BW; DM+FPH2: Diabetic rats treated with fish protein hydrolysate dosage 1.6 g/kg BW. [abcde] Different superscript letters indicated significant differences ($p < 0.05$).

Effects of fish protein hydrolysate on levels of TNF-α in serum

The measurement of TNFα levels in the serum using ELISA resulted in the DM rats group having the highest TNFα levels and significantly different from other groups (Graph 4, $p < 0.05$). Treatment using drug control and FPH decreased the levels of TNFα in the serum significantly compared with DM rats ($p < 0.05$). Moreover, these treatments also improved TNFα levels almost similar to the normal group. This result suggested that treatment using FPH can reduce the pro-inflammatory cytokine TNFα in diabetic conditions.



Graph 4. The levels of TNFα in rats' serum samples. Normal: normal control rats; DM: STZ-induced diabetic rats; DM+FPH1: Diabetic rats treated with fish protein hydrolysate dosage 0.8 g/kg BW; DM+FPH2: Diabetic rats treated with fish protein hydrolysate dosage 1.6 g/kg BW. [abcd] Different superscript letters indicated significant differences ($p < 0.05$).

DISCUSSION

Diabetes mellitus is a global health concern, recognized as the seventh leading cause of mortality worldwide (Li et al., 2023). Diabetes mellitus, as a chronic metabolic disorder characterized by elevated blood glucose levels, poses significant health challenges for individuals worldwide (Shah et al., 2022). Moreover, the consumption of a high-fat diet (HFD) and changes in lifestyle lead to obesity in society. Obesity is identified as the most significant risk factor for the development of insulin resistance, which induces type 2 DM (T2DM; Ruze et al., 2023). A High-Fat Diet causes alterations in plasma membrane cholesterol and insulin binding and signaling (Sabapathy et al., 2022). Insulin facilitates glucose transport from the bloodstream into adipose cells and muscle via insulin receptors (Chadt and Al-Hasani, 2020). Accumulation of ectopic fat has an impact on insulin sensitivity, thus leading to high levels of glucose in the bloodstream (Merry et al., 2020). Obesity also contributed to impairing the antioxidant defense system, reducing the effectiveness of antioxidants like superoxide dismutase (SOD), catalase, and glutathione peroxidase leading to excessive oxidative stress (Čolak et al., 2020).

Researchers have been exploring alternative approaches to manage this menace, including the utilization of natural products (Olasehinde et al., 2021). Fish processing generates significant amounts of by-products, such as frames, bones, skins, and tails, which can be valuable sources of high-quality proteins and bioactive compounds (Caruso et al., 2020). Recent studies have highlighted the potential of bioactive hydrolysates and peptides derived from various food sources, including fish by-products (Kehinde and Sharma, 2020; Daskalaki et al., 2023; Ghalamara et al., 2024). Fish processing by-products have been reported as rich sources of bioactive material such as enzymes, polyunsaturated fatty acids (PUFA), collagen, gelatin, vitamins, minerals, and bioactive peptides (Le Gouic et al., 2018). Fish protein hydrolysate has gained attention for its potential therapeutic benefits, particularly in managing diabetes (Wan et al., 2023). Fish Protein Hydrolysate is produced by the enzymatic hydrolysis of fish proteins, resulting in a mixture of peptides and amino acids that exhibit various bioactive properties (Jafar et al., 2024). The hydrolysis process breaks down proteins into smaller peptides and amino acids, which are easier to digest and absorb (Abraha et al., 2017; Suma et al., 2023).

Skipjack tuna is a widely consumed fish species that generates significant by-products during processing, which may have untapped therapeutic potential (Ramu et al., 2022). Hydrolyzed proteins and peptides isolated from skipjack by-products have been the focus of growing scientific interest due to their reported anti-diabetic properties (Kehinde and Sharma, 2020). Based on the result of this study, the FPH from skipjack by-products decreased blood glucose levels significantly in diabetic rats. Fish protein hydrolysate might have had the ability to inhibit enzymes that are involved in carbohydrate metabolism, such as α-amylase and α-glucosidase. This result is supported by a previous study where FPH using papain enzyme resulted in the effective inhibition of α-amylase (Sarteshnizi et al., 2021). Inhibition of α-amylase can manage the regulation of blood glucose levels (Kehinde and Sharma, 2020). By hindering the breakdown of complex carbohydrates into simpler sugars, these hydrolysates can potentially help mitigate postprandial hyperglycemia, a common challenge faced in the development of diabetes. Fish protein hydrolysate also has been reported as a function to increase insulin sensitivity (Wan et al, 2023). The bioactive peptides in FPH can enhance insulin sensitivity by modulating insulin signaling pathways, leading to improved glucose uptake by cells thus improving the blood glucose levels (Daskalaki et al., 2023). The mechanism of bioactive peptides from FPH to insulin resistance modulation by stimulating the secretion of glucagon-like peptide-1 (GLP1), which has a function to produce more glucose-dependent insulin from pancreatic β-cells (Elbira et al., 2024). On the other hand, the FPH peptides also act as inhibitors of dipeptidyl peptidase-IV (DPP4) activity, leading to increased glucose uptake and reduced blood glucose levels (Zhou et al., 2021).

On the other hand, DM significantly impacts the immune system and promotes chronic inflammation, leading to an increased risk of infections and diabetes-related complications (Gofur et al., 2024). Inflammation is associated with an increase in the production of pro-inflammatory cytokines and activation of leukocytes (Vaibhav et al., 2024). The findings revealed that the DM rats had the largest counts of leukocytes, lymphocytes, and elevated levels of the pro-inflammatory cytokine TNFα. After treatment, FPH showed a lowering in leukocytes, lymphocytes, and the levels of TNF-α (Graphs 3 and 4). Fish protein hydrolysate contains peptides with high antioxidant properties, which can decrease oxidative stress and lead to oxidative damage in the tissue and organ (Wan et al., 2023). Fish protein hydrolysate can protect pancreatic β-cells and enhance insulin production by reducing the effects of oxidative damage (Nikoo et al., 2023). Furthermore, it was reported that FPH supplementation has been shown to improve blood glucose levels, increase insulin sensitivity, and reduce oxidative stress and inflammation (Kehinde and Sharma, 2020). Oxidative stress is a major factor in the pathogenesis of diabetes, and the improvement of oxidative stress can repair the inflammation status, including leukocyte, lymphocyte, and pro-inflammatory cytokine TNF-α (Gambini and Stromsnes, 2022).

CONCLUSION

Fish protein hydrolysate (FPH) from skipjack tuna by-product ameliorates the blood glucose levels, improving the immune system activity and inflammatory markers in STZ-NA-induced diabetic rats. The hydrolysis process in fish by-products gains beneficial values, especially for anti-diabetic candidates. Further research on the benefits of FPH in various metabolic syndrome diseases is necessary to explore the function and benefits of skipjack tuna by-product protein hydrolysate.

DECLARATIONS

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Authors' contributions

Dwi Yanuar Budi Prasetyo carried out the *in vivo* animal model, performed analysis, and drafted the manuscript. Tri Winarmi Agustini participated in the study design and drafted the manuscript. Gemala Anjani participated in its design and coordination and helped draft the manuscript. Putut Har Riyadi performed fish hydrolysis and study design and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declared no conflict of interest.

Ethical considerations

This paper was originally written by the authors and has not been published elsewhere. The authors checked the text of the article for plagiarism index and confirmed that the text of the article is written based on their original scientific results.

Availability of data and materials

The original data presented in the study are included in the article. Data are available from the corresponding author upon reasonable request.

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