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Structural Characteristics of *Theragra chalcogramma* Milt Peptides and Their Anti-Fatigue Activity via AMPK/PGC-1αMediated Energy Metabolism Regulation in Exercised Mice

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Abstract

Objectives: While several physiological functions of milt peptides have been discovered, the structural characteristics of *Theragra chalcogramma* milt peptides (TMP) and their anti-fatigue mechanisms remain unclear. **Methods**: TMP was obtained by hydrolysis via flavor enzyme and alkaline protease, and its structural characteristics were analyzed. A mice model of exercise-induced fatigue was established. The anti-fatigue effect of TMP was evaluated by determining the main biochemical indices in the serum, liver, and skeletal muscle of mice. Additionally, qPCR analysis was conducted to investigate its regulatory effects on relevant energy metabolism pathways. **Results**: TMP contained 18.2% branched-chain amino acids, with those with molecular weights below 1000 Da accounting for 91.6%. A total of 154 characteristic peptides, such as VPFPR and LPPGR, were identified from TMP, among which 64% of the peptides contained glutamic acid, arginine, or aspartic acid. Molecular docking of potential bioactive peptides to AMP-activated protein kinase (AMPK) revealed binding energies from −9.1 to −5.5 kcal/mol. The exhaustive swimming test showed that oral administration of TMP prolonged the swimming duration. In the fatigue murine model, TMP reduced blood urea nitrogen and blood lactic acid levels while enhancing the content of muscle glycogen. Meanwhile, TMP significantly increased the activity of glutathione peroxidase and superoxide dismutase and reduced the accumulation of malondialdehyde, demonstrating antioxidant properties. Additionally, TMP significantly decreased creatine kinase and lactate dehydrogenase extravasation, thereby protecting muscle tissue, as corroborated by immunohistochemical analyses. Mechanistically, TMP upregulated AMPK and peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) expression, promoting mitochondrial biogenesis via the AMPK/PGC-1α pathway. **Conclusions**: These findings suggest TMP has potential as a dietary supplement for alleviating physical fatigue.

Keywords: milt; peptide; anti-fatigue; identification; energy metabolism

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1. Introduction

Exercise fatigue, referred to as muscle fatigue, is usually defined as a physiological phenomenon of a sustained decrease in the body's working capacity due to muscle movement beyond its limits [1,2] when an organism undergoes a period of strenuous physical exertion and it is unable to maintain a physiologically normal level [3]. Fatigue often occurs due to a combination of factors, and currently, research focuses on fatigue-producing mechanisms such as oxidative stress, metabolite accumulation, and energy depletion [4]. Among these, AMP-activated protein kinase (AMPK) plays a pivotal role in the regulation of energy metabolism [5]. Simultaneously, serving as an upstream protein, it positively regulates peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) and mitochondrial transcription factor A (TFAM) [6], thereby promoting mitochondrial biogenesis and enhancing cellular energy supply capacity [7]. Moreover, prolonged untreated fatigue can lead to chronic damage across multiple bodily systems, including cognitive decline and compromised immune function [8]. Some currently available anti-fatigue drugs can cause adverse cardiovascular and neurological effects [9], prompting growing interest in natural alternatives with fewer side effects [10].

Peptides with bioactivity obtained by enzymatic degradation of proteins have been shown to exert anti-fatigue effects in addition to polysaccharides, polyphenols, alkaloids, etc. [11]. These bioactive peptides come from a variety of sources and can be obtained from different plants and animals, such as sea horse peptides [12], loach peptides [13], soybean peptides [14], etc. Among these sources, marine organisms represent a significant reservoir of anti-fatigue peptides [15]. Wang et.al. revealed that sea cucumber peptides with different degrees of hydrolysis were able to significantly enhance mitochondrial function and improve the endurance capacity of mice by the activation of the nuclear factor erythroid 2-related factor 2 (NRF2) and AMPK signaling pathways [6]. Similarly, the results of Xu et.al. showed that hydrolysates from monkfish liver notably elevated SOD activity in vital organs and reduced fatigue symptoms in mice [16].

Theragra chalcogramma is an economically important fish caught in distant water, and its processing generates waste of about 40% of the fish's weight, of which milt accounts for about 7% [17], but the fishy odor is unacceptable, and the utilization rate is low. In fact, *Theragra chalcogramma* milt (TM), a critical visceral component of male fish, is rich in essential amino acids, nucleic acids, and other nutrients [18,19]. Milt-derived peptides have also demonstrated a range of health advantages, such as enhancing insulin sensitivity and combating obesity in mice fed high-fat diets [20], as well as reducing blood pressure by inhibiting ACE activity [21]. Furthermore, basic amino acids from milt such as arginine and lysine can reduce blood ammonia, promote recovery, and delay fatigue [22]. Additionally, studies have shown that milt peptides exhibit antioxidant activity by activating antioxidant enzymes, which can clear free radicals and reduce malondialdehyde levels [23]. This antioxidant capacity may be an effective strategy to alleviate physical fatigue.

In this study, the structure and anti-fatigue activity of *Theragra chalcogramma* milt peptides (TMP) was characterized and predicted by UHPLC-Q-Orbitrap and molecular docking. The effect of peptides on alleviating exercise fatigue was evaluated by establishing a forceful and isochronous swimming model using blood and muscle biochemical parameters, muscle tissue morphology, and so on. Further, their role in energy metabolism was explored through the AMPK/PGC-1α signaling pathway.

2. Materials and Methods

2.1. Materials

Theragra chalcogramma milt (TM) was procured from Liaoyu Group Co., Ltd. (Dalian, China). Alkaline protease, trypsin, neutral protease, flavor enzyme, and papain were

provided by Shanwan Biotechnology Co., Ltd. (Nanning, China). A malondialdehyde (MDA) assay kit (A003-1), a glutathione peroxidase (GSH-Px) assay kit (A005-1), a superoxide dismutase (SOD) assay kit (A001-1), a blood lactic acid (BLA) assay kit (A019-2), a blood urea nitrogen (BUN) assay kit (C013-2), a lactate dehydrogenase (LDH) assay kit (A020-2), a creatine kinase (CK) assay kit (A032-1), an ATP assay kit (A095-1), an ATPase assay kit (A016-2), and muscle glycogen (MG) and liver glycogen (LG) assay kits (A043-1) were manufactured by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). A bicinchoninic acid protein concentration kit (P0010) was purchased from Biyuntian Institute of Biotechnology (Shanghai, China); the chromatography and mass spectrometry reagents were of chromatographic grade. Other reagents were of analytical grade.

2.2. Preparation of TMP

TM was mixed and homogenized with pure water at a 1:10 (*w*/*w*) ratio. This homogenized solution was then subjected to enzymatic hydrolysis by the addition of papain (Pap), alkaline protease (Alk), neutral protease (Neu), flavor enzyme (Fla), and trypsin (Try). TM was subjected to treatment at an enzyme-to-substrate ratio (E/S, *w*/*w*) of 0.5–2.5% at 55 °C for 4 h. The enzyme-treated solution was heat-inactivated at 95 °C for 10 min to terminate enzymatic reactions and then subjected to centrifugation at 10,000 rpm for 20 min, concluding with lyophilization. Based on the results of one-way experiments, two enzymes were screened to hydrolyze TM at enzyme-to-enzyme ratios (E/E, *w*/*w*) of 1:5, 1:2, 1:1, 2:1 and 5:1.

2.3. The Degree of Hydrolysis and Nitrogen Recovery

Nitrogen recovery (NR) and the degree of hydrolysis (DH) were used as screening indices for enzyme selection. DH was assessed by ninhydrin colorimetry, and NR was determined by the Kjeldahl method for the hydrolysates [24].

2.4. Characteristics of TMP

2.4.1. Determination of Molecular Weight Distribution

TMP's molecular weight was determined via HPLC (Agilent 1200, Agilent Technologies, Inc., Santa Clara, CA, USA) with a TSK gel G2000SWXL column (300 × 7.8 mm, Tosoh Bioscience, Tokyo, Japan), detected at 225 nm. The mobile phase consisted of a mixture of water, trifluoroacetic acid, and acetonitrile in a ratio of 700:1:300 (*v*/*v*/*v*) [25].

2.4.2. FT-IR

The transmittance of TMP was measured over a wavenumber range of 4000 to 400 cm^{-1}. To prepare the samples, TMP was mixed and pressed with dried KBr to form tablets, which were subsequently scanned 64 times.

2.4.3. Circular Dichroism Spectroscopy (CD)

The CD spectrum of TMP (0.05 mg/mL) was measured on a spectropolarimeter (J-1500, JASCO Co., Tokyo, Japan) within a scanning range of 190–260 nm. The scanning speed was 200 nm/min, and the D.I.T was 1 s.

2.4.4. Peptide Fragment Sequences of TMP

Samples were analyzed using an Ultimate 3000 liquid mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a C18 column. The mass spectrometry conditions were set as follows: spray voltage of 3600 V, Full MS-ddMS2 scanning mode, scanning range of 100–1200 m/z, positive ion mode, and capillary temperature of 320 °C. The data were analyzed using Peaks Online 1.7 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) [26].

2.5. Molecular Docking of TMP with AMPK

The fatigue-reduction potential of TMP was explored through molecular docking studies, following the methodology outlined by Qi et al. [27] AMPK's 3D structure (PDB ID: 4ZHX) was accessed via the PDB database (https://www.rcsb.org/ (accessed on 11 March 2025)). ChemDraw 19.0 was used to draw the peptide structure, and Chem3D 19.0 was applied to minimize its energy. Peptides served as the ligand, with AMPK as the receptor, docked using AutoDockTools 1.2.5. The docking was performed using AutoDock Vina with the grid box set at center coordinates (525.356, −2.775, 1016.793) and size (27.7, 58.1, 56.7). The results were processed using PyMOL v2.6 and Discovery Studio v19.1.0.

2.6. Experimental Animals and Treatment

Sixty healthy Balb/c mice, SPF grade, male (6 weeks old, 18–22 g) were purchased from Pengyue Laboratory Animal Breeding Co Ltd. (Jinan, China). Mice were housed in a 12 h light—dark environment at 23 ± 2 °C. After acclimatization for one week, mice were subjected to three swimming screening sessions. During screening, mice were allowed to swim freely in tanks (depth ≥ 30 cm, 25 ± 2 °C). Those with a swimming time of less than 5 min were considered abnormal and rejected [28]. The remaining mice were divided randomly into five groups of 10 each [28,29], categorized as follows: (1) resting control group (Rest, saline), (2) swimming control group (Con, saline), (3) positive control group (PC, 500 mg kg^{-1} d^{-1} taurine), (4) high-dose group (TMP-H, 800 mg kg^{-1} d^{-1}), and (5) low-dose group (TMP-L, 400 mg kg^{-1} d^{-1}). In anti-fatigue research using murine models, the effective doses of many bioactive peptides typically fall within these ranges [30,31]. The interventions lasted 30 days, and the order of daily oral gavage administration was systematically rotated among the different experimental groups each day. Using the standard approach to convert based on body surface area, the human equivalent dose of TMP was estimated to be roughly 32.43 to 64.86 mg kg^{-1} d^{-1} [32]. For an adult weighing 60 kg, this translates to a daily intake of approximately 1.95 to 3.89 g. For humans, TMP can be formulated into a mixture and brewed for intake, similar to a solid beverage, or formed into oral tablets to be swallowed.

After a 30 min interval following the last administration, mice, except those in the Rest group, were compelled to swim unloaded for 30 min within the tanks (depth ≥ 30 cm, 25 ± 2 °C). During testing, a glass rod was employed to ensure the mice swam continuously [33]. Following the swim session, blood samples were collected, and the mice were immediately humanely killed by cervical dislocation. Among them, 4 mice were randomly selected for histological staining, and the remaining 6 mice were used for biochemical and molecular biological analyses. A single-blind design was used in this study. The liver and gastrocnemius and quadriceps femoris muscles were then rapidly harvested, flash-frozen in liquid nitrogen, and stored at −80 °C until further analysis. All animal testing conducted in this study strictly followed internationally recognized ethical guidelines for animal welfare. Furthermore, the above work received approval from the Ocean University of China's Animal Ethics Committee (Approval No. SPXY2023100830) on 08-10-2023.

2.7. Exhaustive Swimming Test

After a 30 min interval following the last administration, mice, except those in the Rest group, were subjected to the exhaustive swimming test on day 24. The animals were forced to keep swimming in tanks filled with water (depth ≥ 30 cm, 25 ± 2 °C) with a piece of lead (5% of their weight) tied to their tails, and failure to float for 7 s with the head submerged in the water was considered exhaustion [34]. The time to exhaustion was calculated from the start of swimming until this exhaustion threshold was reached.

2.8. Measurement of the Serum Biochemical Parameters

Blood samples were centrifuged (6000 rpm, 4 °C, 40 min) after 30 min of incubation to isolate serum. Using standard assay kits, MDA, GSH-Px, SOD, BLA, BUN, LDH, and CK were then measured.

2.9. Measurement of Liver and Muscle Biochemical Parameters

The gastrocnemius muscle was accurately weighed (0.1 g), mixed with 0.9 mL of ice-cold saline, homogenized at 60 Hz for 50 s per cycle (6 cycles in total) at 4 °C, and then centrifuged. The supernatant was used to measure Na^+-K^+-ATPase, Ca^{2+}-Mg^{2+}-ATPase, ATP, LDH, CK, SOD, GSH-Px, and MDA. Similarly, the quadriceps femoris muscle and liver were homogenized, and the resulting extracts were used to determine MG and LG levels by kits.

2.10. Histological Study

The fixed left gastrocnemius muscle of mice was embedded with paraffin. After the aforementioned procedures, the tissues were sectioned into 5 μm slices and stained using hematoxylin and eosin (H&E) [35]. Subsequently, pathological changes were examined through a microscopic imaging system.

2.11. Real-Time qPCR

Muscle tissue samples were processed for total RNA extraction using TRIzol reagent, with RNA concentrations measured precisely via the Nano-100 micro spectrophotometer (HINOTEK Group Ltd., Ningbo, China). Following extraction, the RNA was converted into complementary DNA through reverse transcription. Primer sequences, detailed in Table S1, were employed for subsequent qPCR analysis. Amplification was carried out on a LineGene 9600 Plus PCR system (HINOTEK Group Ltd., Ningbo, China) using SYBR Green Master Mix for fluorescence detection.

2.12. Enzyme-Linked Immunosorbent Assay (ELISA)

Protein expression levels of AMPK and PGC-1α were analyzed using ELISA kits purchased from Suzhou Calvin Biotechnology Co., Ltd. (Suzhou, China).

2.13. Statistical Analysis

The data were expressed as means ± SD. Figures were created with GraphPad Prism 9.5 and Origin 2021. The data were analyzed by one-way ANOVA, with Duncan's post hoc tests, using IBM SPSS Statistics 26.

3. Results and Discussion

3.1. Screening of Enzyme Digestion Conditions of TMP

As shown in Figure 1A, TM exhibits a comprehensive amino acid profile with a high proportion of branched-chain amino acids (BCAA, 18.4%), including Leu (9.0%), Val (5.8%), and Ile (3.7%). In enzymatic digestion, NR and DH are key indices reflecting raw material utilization and digestion efficiency, respectively [36]. These two parameters collectively indicate the effectiveness of enzymatic treatment. NR and DH under different conditions are shown in Figure 1B–F, and the results showed that DH and NR gradually increased with the increase in enzyme addition across the five enzymes. NR and DH of samples treated with Alk and Fla were higher than those of samples treated with the other three enzymes, and NR basically stayed above 80%, with Alk having the highest NR of up to 91.43 ± 1.75%. For comprehensive consideration, Alk and Fla were selected for complex enzymatic hydrolysis. This is consistent with the view of Yue et al., i.e., alkaline protease

and flavor enzyme can be used to hydrolyze silver carp steak to produce antioxidant peptides [37].

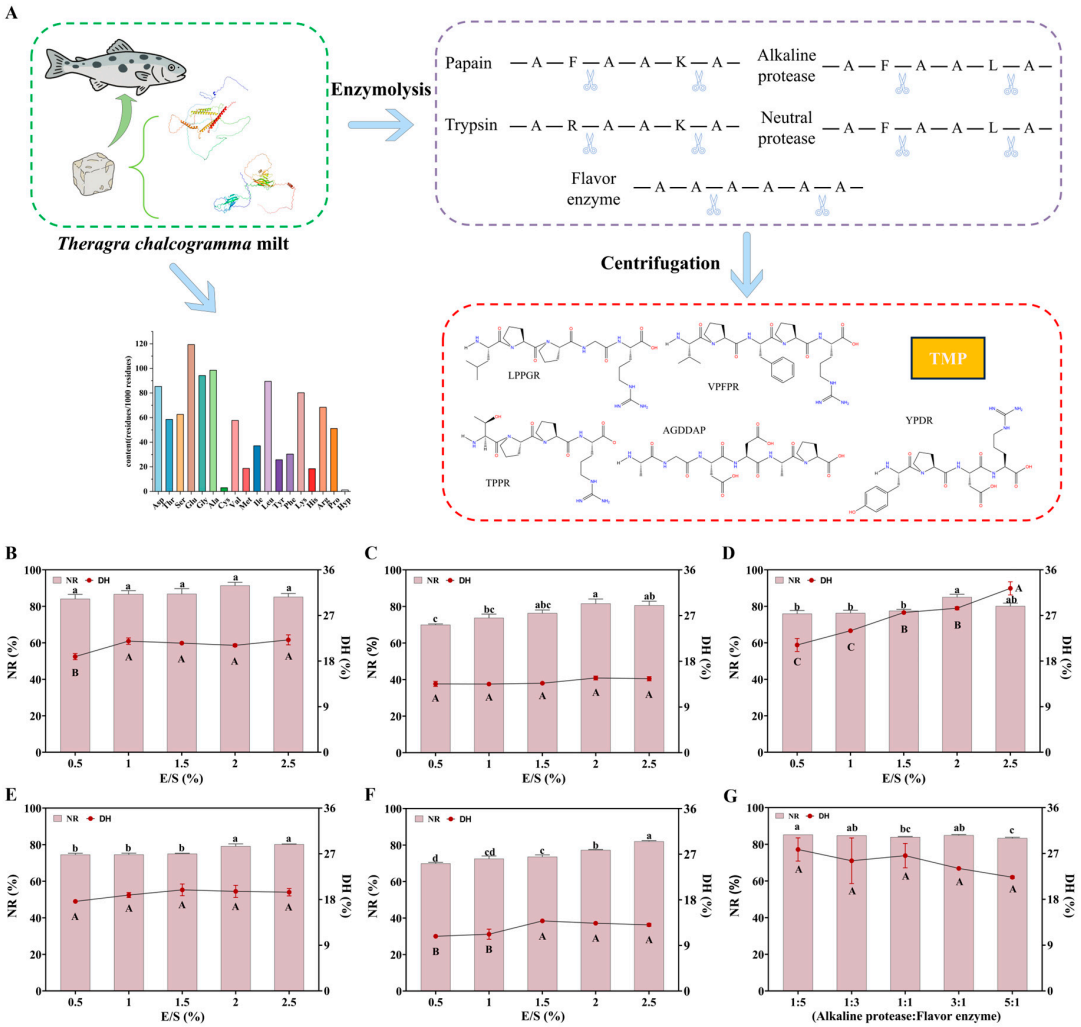

Figure 1. (**A**) *Theragra chalcogramma* milt enzymatic hydrolysis flowchart. Nitrogen recovery (NR) and the degree of hydrolysis (DH) of enzymatic hydrolysates under different conditions. (**B**) Alkaline protease (Alk), (**C**) papain (Pap), (**D**) flavor enzyme (Fla), (**E**) trypsin (Try), (**F**) neutral protease (Neu). (**G**) NR and DH of complex enzymatic hydrolysis. Different lowercase letters indicate significant differences in NR, and different uppercase letters indicate significant differences in DH, $p < 0.05$.

According to the results of NR and DH of single enzymatic digestion, Alk and Fla were selected for further optimization of the enzymatic digestion conditions. The hydrolysis was performed at pH 8 and 55 °C for 4 h. As shown in Figure 1G, when the enzyme addition ratio of Alk and Fla was 1:5, DH was 27.7 ± 2.2% and NR was 85.2 ± 0.0%. This value was the highest among the enzyme addition ratios, so the enzyme hydrolysis product under this condition was defined as TMP, and the structure and activity of the product were subsequently explored.

3.2. Structural and Physicochemical Characteristics of TMP

The molecular weight of peptides is a critical determinant of their biological activity. Specifically, peptides with low molecular weights tended to demonstrate elevated bioactivity due to enhanced membrane permeability and target interaction [36,38]. As shown in Figure 2A, the molecular weight distribution of TMP was as follows: most of the peptides had molecular weights of 200–600 Da, and 91.6% of the peptides in TMP were

below 1000 Da. In Figure 2B, the amino acid analysis showed that BCAA made up 18.2% of the amino acids, and some researchers have suggested that BCAA reduces protein loss during strenuous exercise and speeds up the body's recovery [39]. Similar to Zhao et al.'s research, it was also observed that whey protein hydrolysate rich in BCAA modulated energy metabolism and mitigated mice exercise-induced injury [40]. In addition, TMP was also rich in Glu (11.4%), Arg (6.2%), and Asp (7.2%). Glu exhibited advantages during physical activity and enhanced the nervous system's function [41]. Asp participated in the urea cycle, thereby reducing circulating ammonia levels and alleviating ammonia-induced fatigue [42]. Arg was also regarded as a key amino acid for delaying fatigue and antioxidation [31]. The above findings demonstrated that TMP might exhibit anti-fatigue properties.

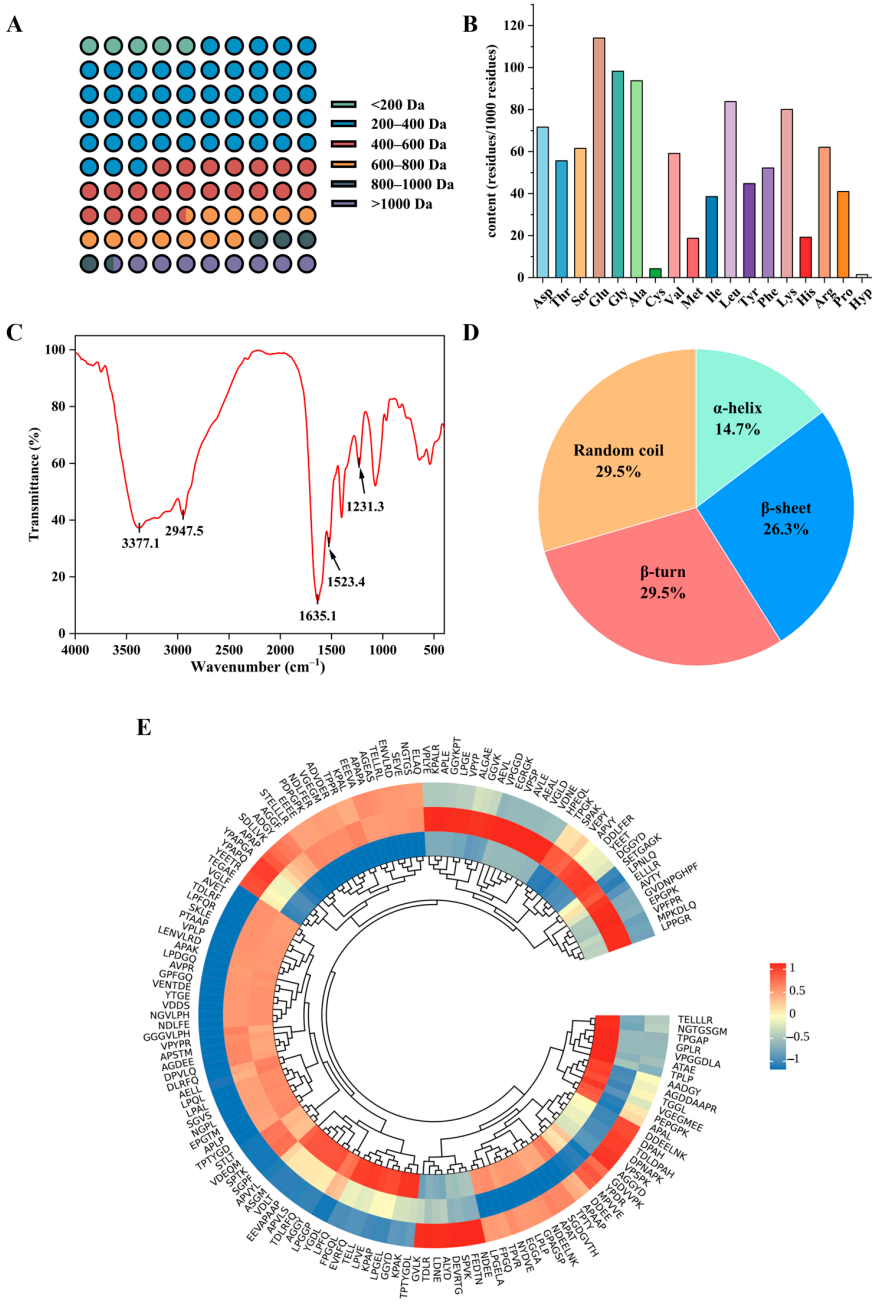

Figure 2. Characteristics of *Theragra chalcogramma* milt peptides (TMP). (**A**) Molecular weight distribution, (**B**) amino acid composition, (**C**) FT-IR spectrum, (**D**) ratio of secondary structures, and (**E**) major characteristic peptide sequences.

The FT-IR results are demonstrated in Figure 2C, in which the amide A band was located at a wave number of 3377.1 cm^{-1}, indicating the stretching vibrations of –NH and –OH [43]. A distinct narrow absorption peak at 2947.5 cm^{-1} was indicative of asymmetric $-CH_2$ stretching vibrations. A characteristic peak of the amide I band of TMP was detected at 1635.1 cm^{-1}, arising from the stretching vibration of C=O within the peptide bonds [44]. In addition, absorption bands at 1523.4 cm^{-1} and 1231.3 cm^{-1} were assigned to the amide II and III bands, respectively. In the sections that follow, the results of the CD spectroscopy analyzed by the online analysis software Dichroweb (https://dichroweb.cryst.bbk.ac.uk/html/home.shtml (accessed on 8 May 2025)), are exhibited in Figure 2D [45]. This figure elucidated that TMP was mainly composed of irregular curls (29.5%) and β-turns (29.5%), in addition to 14.7% of helices and 26.3% of β-folds. The above characteristics unveil the nature of TMP.

Peptide mass fingerprinting analysis of TMP revealed the identified peptide segments, as presented in Figure 2E, with a total of 154 recurring peptides (confidence interval > 95%). These listed sequences had a length of 4–9 amino acids, of which 43 peptides were more likely to exhibit biological activity (peptide ranker response > 0.5) [46]. In addition, 64% of the peptide segments contained Glu, Arg, and Asp.

3.3. Molecular Docking of Peptides with Anti-Fatigue Activity with AMPK

Molecular docking simulation serves as a computational tool commonly employed to predict the binding affinity between ligands and proteins [47]. AMPK is a trimeric complex that participates in signaling pathways related to energy metabolism by binding AMP, ADP, and ATP via the γ-subunit [48]. Docking studies of AMPK have been used to predict the anti-fatigue activity of hydrolysates and peptides [49,50].

Molecular docking of potentially bioactive peptides predicted from TMP with AMPK was performed as a supportive, hypothesis-generating approach to explore their possible anti-fatigue activity. The greater the binding energy's absolute value, the stronger the indication of receptor–ligand interaction. Furthermore, binding energies less than −5.0 kcal/mol are generally regarded as being stable [51]. The binding energies for these 43 peptides with AMPK ranged from −9.1 to −5.5 kcal/mol (AMPK Binding affinity in Table 1), indicating stable interactions between the receptor and the ligands. To further analyze the interaction of the peptides with AMPK receptors, two peptides, TPPR and YPDR, which contained Arg and exhibited relatively high binding affinities, were selected for analysis of their interaction force and optimal conformation as presented in Figure 3.

The peptides TPPR and YPDR could be effectively embedded within the active pocket of AMPK, where they docked into the γ-subunit primarily through van der Waals forces and hydrogen bonding. Figure 3B shows that TPPR formed hydrogen bonds with Asp317, Ser316, Ala205, Ala227, Arg299, Ser226, and Val225 in the AMPK γ-subunit; in addition, it formed alkyl bonds with His151 and Lys149. Figure 3D shows that YPDR formed hydrogen bonds with Ser316, Ser226, and Thr200 in the AMPK γ-subunit; furthermore, it formed alkyl bonds with Ile312, Ile204, and Val225 while also forming a single attractive charged interaction with Asp317. These specific amino acids were crucial for the interactions between AMP and the AMPK γ-subunit.

In summary, TMP has the potential to regulate the expression of downstream signal molecules, including PGC-1α and nuclear respiratory factor 1 (NRF1), by activating AMPK, thereby alleviating fatigue. Molecular docking provided evidence for the potential stable binding of TMP-derived peptides to the AMPK γ-subunit, but these results remain predictive and hypothesis-generating. It is critical to acknowledge that predicted binding affinity does not equate to physiological efficacy. Factors like peptide absorption and

metabolism profoundly impacted their functional outcomes, thus necessitating in vivo studies for definitive verification.

Table 1. The docking energy of potential bioactive peptides with AMP-activated protein kinase (AMPK).

Peptide Sequences	Predicted Activity Values	AMPK Binding Affinity (kcal/mol)	Peptide Sequences	Predicted Activity Values	AMPK Binding Affinity (kcal/mol)
SGPF	0.964201	−8.8	APAL	0.614098	−7.8
AGGF	0.955482	−8.4	VPYP	0.608933	−7.2
FPGQ	0.914028	−8.6	YPAPGA	0.604127	−8.7
FPGQL	0.913499	−8.1	TDLRF	0.596597	−7.3
VPFPR	0.863031	−7.9	VPLP	0.586866	−7.8
GPFGQ	0.823838	−8.1	YPDR	0.578918	−8.8
LPFQ	0.818702	−7.9	LPQL	0.575406	−6.9
LPGGP	0.816817	−7.6	YGDL	0.572756	−8.0
VGLF	0.814525	−7.1	TPPR	0.570039	−8.1
APLP	0.813294	−7.0	TPLP	0.559699	−7.2
GPLR	0.806088	−7.5	DLRFQ	0.558118	−5.5
LPPGR	0.802757	−7.5	APAPA	0.557938	−8.1
LPLP	0.794971	−7.1	GGYD	0.5374	−8.0
NGPL	0.770098	−7.3	YPAPQ	0.53694	−8.3
LPFQR	0.768856	−7.1	KPAP	0.528552	−7.0
GVDNPGHPF	0.71871	−7.1	APVYL	0.526748	−7.6
PDPGPK	0.709132	−7.8	GPAGSP	0.526342	−7.5
APAP	0.70062	−7.1	APAAP	0.522574	−7.4
AGGY	0.694333	−8.5	TPGAP	0.514961	−7.0
LPAL	0.642139	−7.0	AGDDAAPR	0.5056	−9.1
VPYPR	0.641232	−7.9	PEPGPK	0.502534	−8.4
ASGM	0.631677	−6.0			

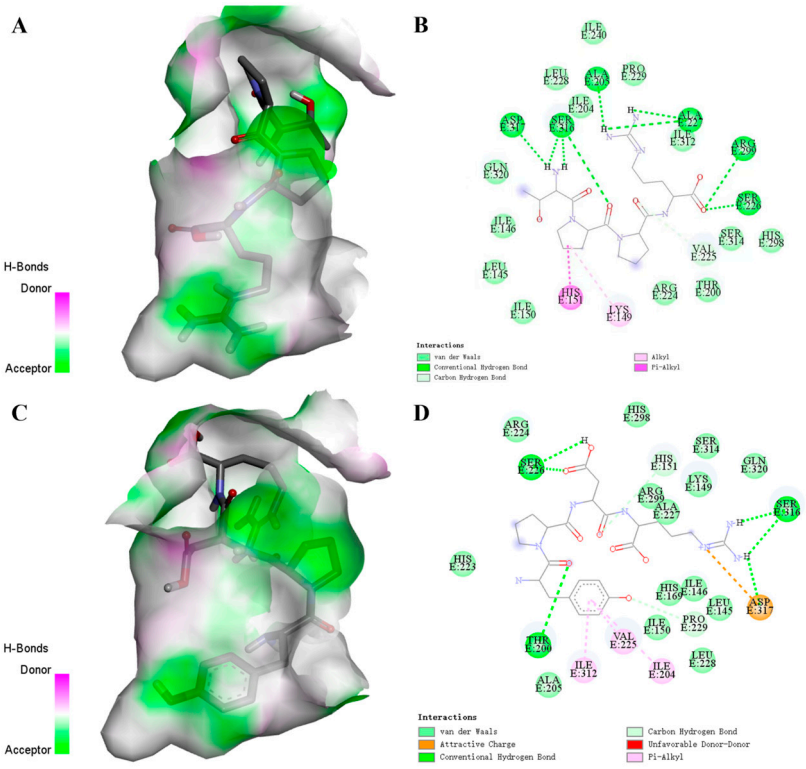

Figure 3. Molecular docking and visualization analysis of representative peptides with AMPK (ID: 4ZHX): (**A**,**C**) Detailed views, and interactions (**B**,**D**).

3.4. Effects of TMP on the Exhaustive Swimming Test

Changes in exercise endurance serve as an indicator of an organism's fatigue level. The exhaustive swimming test is a commonly employed experimental approach to assess the endurance capacity of mice. As shown in Figure 4B, the PC and TMP treatment groups exhibited significantly greater endurance than the control group ($p < 0.01$). Mice in the TMP-L and TMP-H groups showed prolongation of the exhaustion time by 45.7% and 59.2%, respectively. Thus, the findings indicated that TMP could improve exercise capacity in mice. These findings are consistent with earlier research by Lu et al. [52].

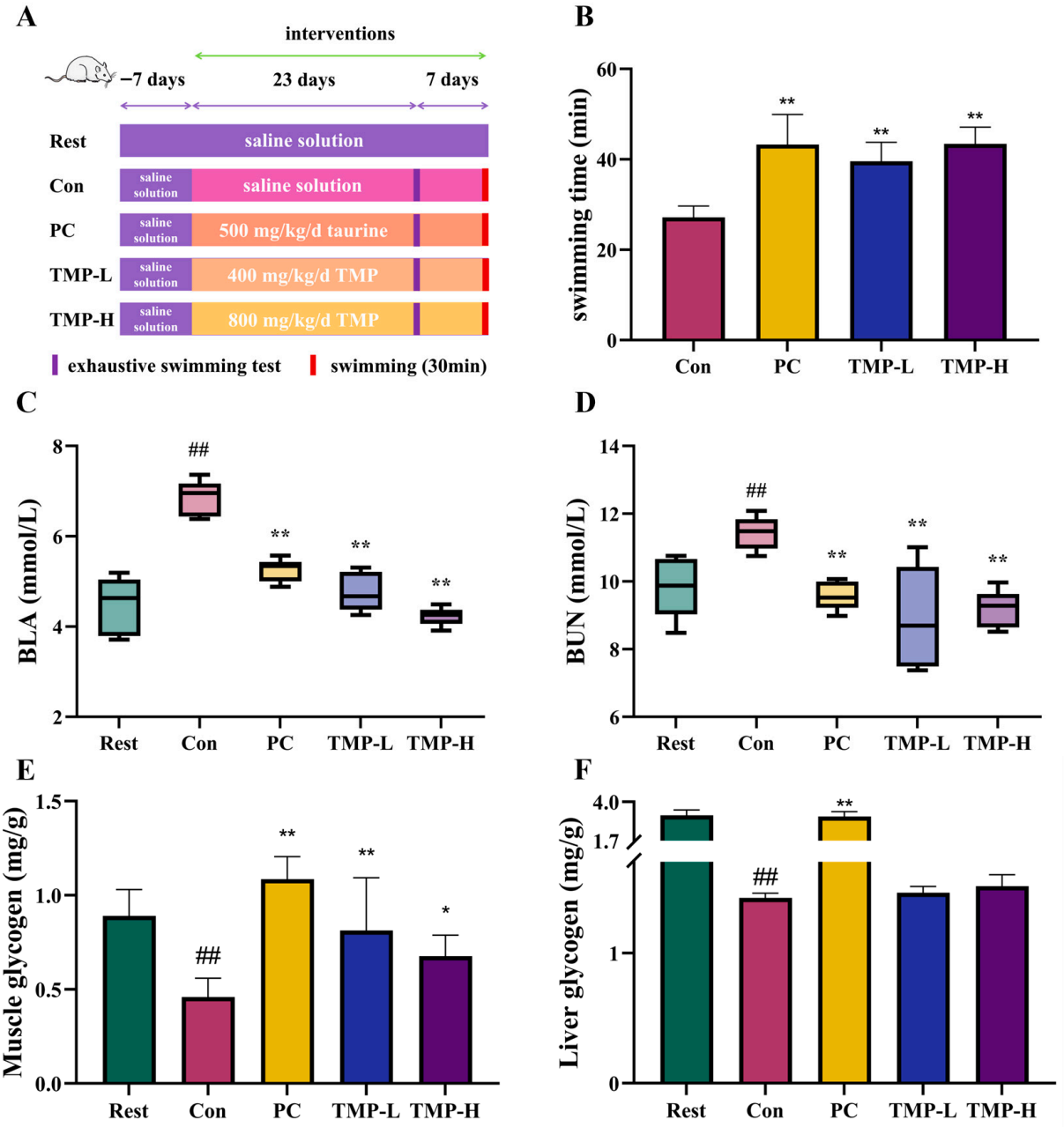

Figure 4. (**A**) Experimental procedure assessing the impact of TMP on fatigue in mice; (**B**) exhaustive swimming time; serum levels of (**C**) blood lactic acid (BLA) and (**D**) blood urea nitrogen (BUN) in mice; concentrations of (**E**) muscle glycogen (MG) and (**F**) liver glycogen (LG) in muscle and liver tissue. ($n = 6$ in each group, ## $p < 0.01$ indicates a significant difference vs. the Rest group, ** $p < 0.01$ indicates a significant difference vs. the model Con group, * $p < 0.05$ indicates a significant difference vs. the Con group.)

3.5. Main Biochemical Indices

In the event of aerobic respiration failing to meet the energy demand, glycolysis becomes the primary pathway for energy acquisition, resulting in the accumulation of BLA [53]. Elevated levels of BLA reduce the pH of muscle tissue, leading to muscle soreness [54]. As shown in Figure 4C, the forced swimming led to a significant elevation in serum levels of BLA ($p < 0.01$). Compared with the Con group, serum levels of BLA were reduced by 38.5% and 30.7% in the TMP-H and TMP-L groups, respectively ($p < 0.01$). BUN serves as a key biochemical marker representing the final product of protein and amino acid catabolism in the human body [55]. In the event of an inadequate energy supply from carbohydrates and fatty acids, the body would consume protein to meet its energy requirements. Consequently, BUN levels are inversely correlated with the body's exercise endurance [56]. As shown in Figure 4D, the serum BUN concentrations were markedly higher in the Con group than in the Rest group ($p < 0.01$). Moreover, both TMP-L and TMP-H groups showed a substantial reduction in serum BUN levels, nearly normalizing, with statistical significance ($p < 0.01$). These findings indicate that TMP may mitigate the accumulation of BUN and BLA in response to exercise.

Glycogen, the primary storage form of glucose, is a crucial source of energy for the body during exercise. As glycogen consumption increases during exercise, glycogen levels become closely related to the body's exercise endurance. As shown in Figure 4E,F, swimming led to a notable reduction of LG and MG concentrations in mice ($p < 0.01$). MG concentrations in the TMP-L and TMP-H groups increased by 76.1% and 45.7%, respectively, versus the Con group ($p < 0.01$ or $p < 0.05$). Conversely, no significant differences were noted among the LG contents across the TMP groups ($p > 0.05$). The differential effect of TMP on glycogen levels may be attributed to the distinct metabolic roles of skeletal muscle and the liver. As the direct site of energy consumption during exercise, skeletal muscle glycogen metabolism is more sensitive to interventions aimed at enhancing exercise endurance [57]. Therefore, TMP was speculated to influence glycogen replenishment efficiency by modulating specific energy metabolism pathways, such as the AMPK pathway, in skeletal muscle. In contrast, LG metabolism, which is centrally governed by systemic hormonal regulation for the maintenance of glucose homeostasis, was likely unaffected by TMP under the present experimental conditions [58]. Unlike the results of this study, Li et al. observed that supplementation with *Brassica rapa* L. extract markedly attenuated the decline in glycogen content [9]. These findings indicate that TMP exerts minimal influence on LG levels when it functions to relieve fatigue.

The impact of TMP on oxidative stress in mice is shown in Figure 5. MDA, the primary byproduct of lipid peroxidation, functions as a reliable biomarker for assessing oxidative damage [59]. SOD and GSH-Px worked synergistically to regulate levels of oxidative stress by neutralizing harmful reactive oxygen species [60]. SOD catalyzes the generation of H_2O_2 and O_2 from ROS. GSH-Px has the capacity to convert toxic peroxides into non-toxic hydroxyl compounds and facilitate the decomposition of H_2O_2 [61]. As shown in Figure 5A,B,D,E, the TMP group showed a marked increase in both SOD and GSH-Px levels relative to the Con group ($p < 0.05$ or $p < 0.01$). As shown in Figure 5C,F, MDA concentrations decreased in both TMP dose groups versus controls, but the reduction in mice muscle did not reach statistical significance ($p > 0.05$). Considering the accumulated findings, it is reasonable to hypothesize that TMP could help alleviate fatigue by enhancing the activity of antioxidant enzymes like SOD and GSH-Px within the body and by inhibiting the formation of lipid peroxides.

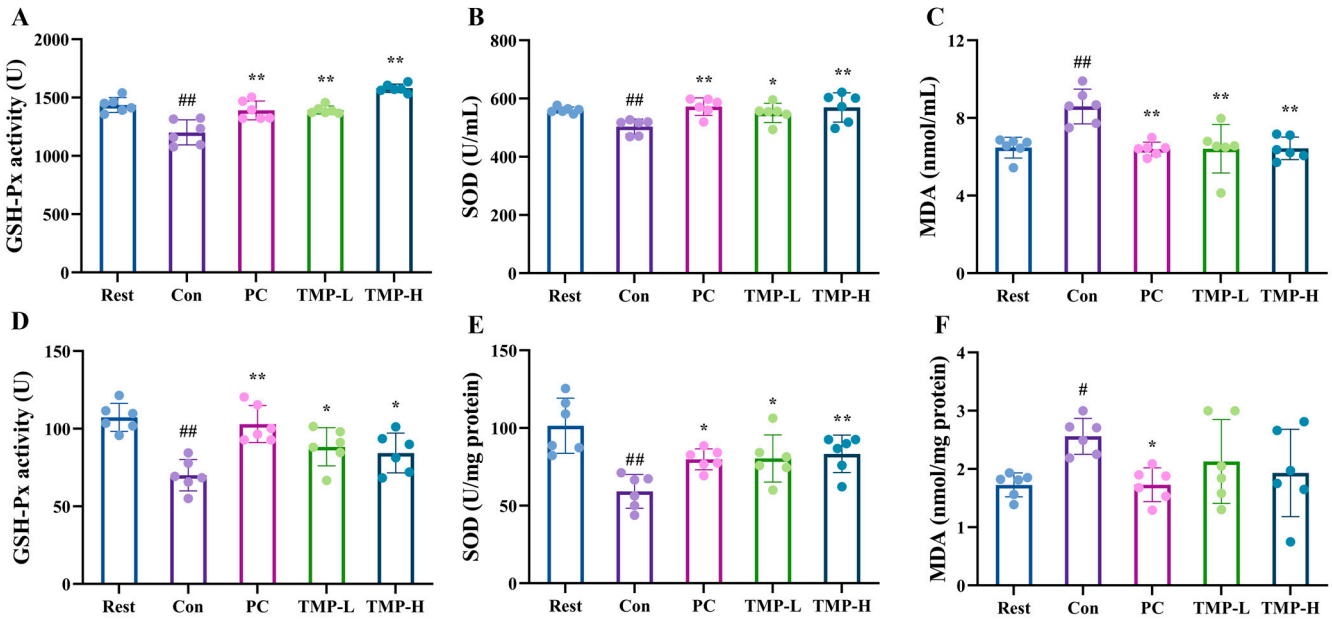

Figure 5. The activity of (**A**) glutathione peroxidase (GSH-Px) and the contents of (**B**) superoxide dismutase (SOD) and (**C**) malondialdehyde (MDA) in mice serum. The activity of (**D**) GSH-Px and the contents of (**E**) SOD and (**F**) MDA in mice muscle. (n = 6 in each group, # $p < 0.05$ and ## $p < 0.01$ indicates a significant difference vs. the Rest group, ** $p < 0.01$ indicates a significant difference vs. the model Con group, * $p < 0.05$ indicates a significant difference vs. the Con group.)

3.6. Effect of TMP on Muscle Injury in Mice

Excessive exercise by an organism might result in damage to the muscle cells [62]. As shown in Figure 6A, the myocytes from the Rest group exhibited a normal morphology, with muscle fibers arranged in an orderly and tight manner. In contrast, the myocytes in the Con group displayed a disorganized arrangement and impaired structural integrity. Relative to the Con group, the PC, TMP-L, and TMP-H groups exhibited varied enhancements in muscle fiber structure, with improved arrangement and structural compactness. The findings indicated that TMP mitigated exercise-induced muscular damage.

Serum CK and LDH are widely recognized markers of muscle damage [63,64]. As shown in Figure 6C,E, there was a notable rise in CK and LDH levels in the serum of the Con group mice versus the Rest group ($p < 0.01$). Treatment with TMP at 800 mg/kg led to a 28.6% decrease in CK and a 25.7% reduction in LDH serum levels compared to the Con group ($p < 0.01$ or $p < 0.05$). It is important to note that when muscle cells were damaged during exercise, the cell membrane permeability increased, allowing CK and LDH from the muscle to enter the bloodstream. TMP administration upregulated the levels of CK and LDH in the muscle (Figure 6B,D). Specifically, the TMP-L treatment significantly increased CK and LDH levels by 17.02% and 24.81%, respectively ($p < 0.01$). The TMP-H treatment increased CK and LDH levels by 9.36% and 13.60%, respectively. These findings further suggested that TMP had potential protective effects against muscle damage during swimming activities. Similarly, the okara protein hydrolysate and the soft-shelled turtle peptides have been shown to reduce CK and LDH, demonstrating anti-fatigue properties [65,66].

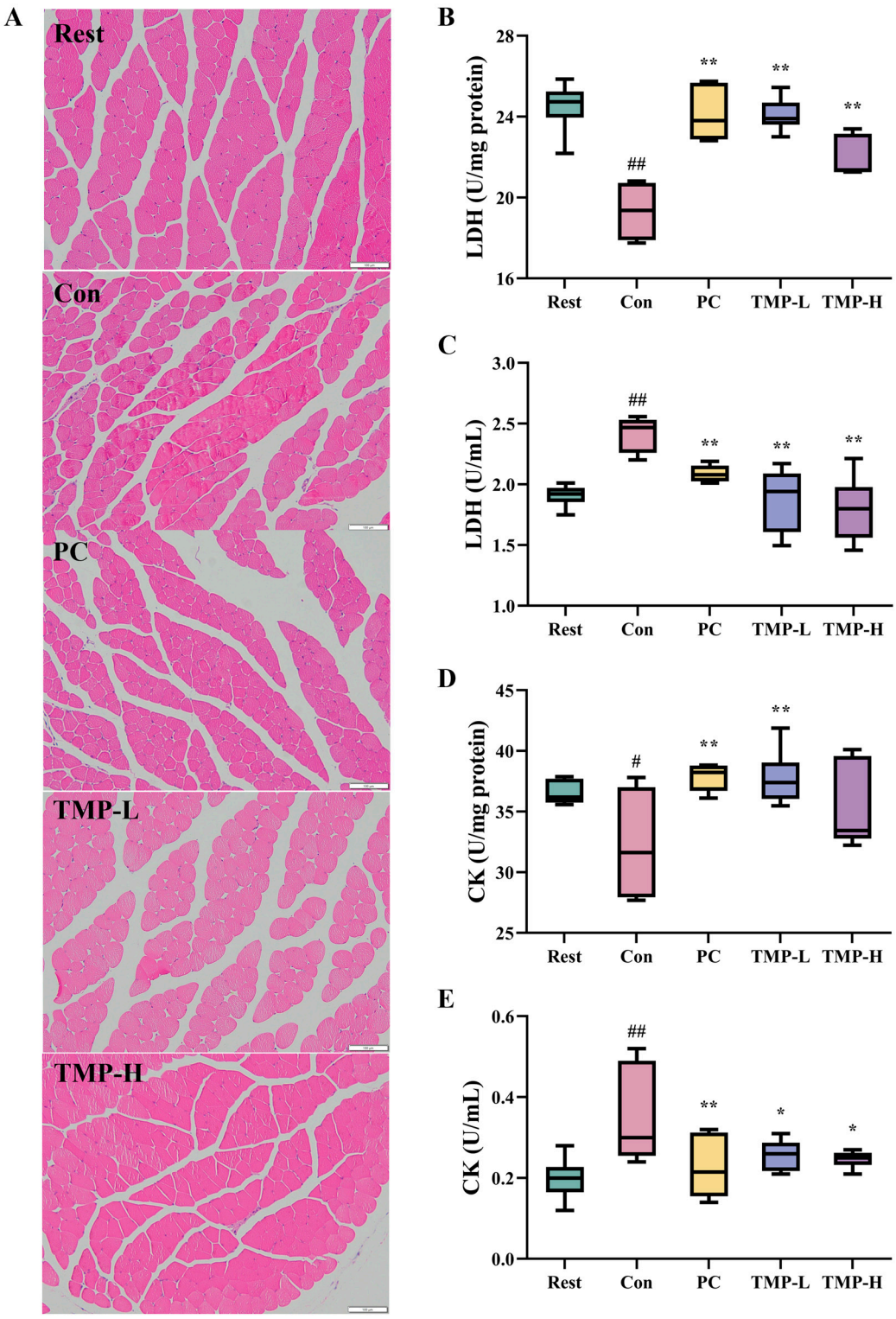

Figure 6. (**A**) H&E staining of the mice gastrocnemius muscle; the contents of (**B**) lactate dehydrogenase (LDH) and (**D**) creatine kinase (CK) in mice muscle; the concentrations of (**C**) LDH and (**E**) CK in mice serum. (n = 6 in each group, # $p < 0.05$ and ## $p < 0.01$ indicate a significant difference vs. the Rest group, ** $p < 0.01$ indicates a significant difference vs. the model Con group, * $p < 0.05$ indicates a significant difference vs. the Con group.)

3.7. Effects of TMP on Fatigued Mice by Modulating Energy Metabolism

Energy metabolism in mice forced to swim was studied by ATP and ATPase (Figure 7). In addition to significantly elevating ATP levels in the skeletal muscle of the TMP-H

group ($p < 0.05$), TMP supplementation also significantly increased Na^+-K^+-ATPase activity ($p < 0.05$ or $p < 0.01$), without affecting Ca^{2+}-Mg^{2+}-ATPase levels ($p > 0.05$). Mitochondria could generate cellular energy in the form of ATP. Under normal physiological conditions, the human body is capable of obtaining an adequate supply of oxygen through aerobic metabolism, thereby producing substantial amounts of ATP [4]. The degradation of ATP is predominantly mediated by two main enzymes: Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase. Of these, Na^+-K^+-ATPase can directly hydrolyze ATP to provide free energy to the organism, while the function of Ca^{2+}-Mg^{2+}-ATPase is primarily that of a calcium ion pump in cell membranes, with the result that muscle contractility is maintained [67]. Zhang et al. similarly found that pigeon meat hydrolysate improves ATPase activity by up-regulating ATPase activity in mice energy band blood, thereby alleviating fatigue [68].

AMPK, a serine/threonine kinase, regulates cellular energy balance by detecting fluctuations in the intracellular levels of ADP, AMP, and ATP [69,70]. Mitochondria also play a crucial role in the regulation of energy metabolism, and PGC-1α is a key regulator that could enhance mitochondrial function [71]. As can be seen from Figure 7D–F,H, compared with the Con group, the mRNA levels of AMPK, PGC-1α, NRF1, and TFAM, except for AMPK in the TMP-L group, were significantly increased ($p < 0.05$ or $p < 0.01$). The upstream target molecule, AMPK, promoted the expression of PGC-1α as well as its downstream signaling, and PGC-1α interacted with NRF1 (a nuclear-encoded transcription factor) and participated in the expression of TFAM, which in turn improved mitochondrial function and regulated energy metabolism [72].

Sirtuin 1 (SIRT1) is a class III deacetylase that relies on nicotinamide adenine dinucleotide (NAD^+) as an essential cofactor. By removing acetyl groups, SIRT1 regulates the activity of key transcription factors, like PGC-1α, ultimately boosting their transcriptional output [73,74]. Additionally, Cytochrome C (Cyt c) is recognized as a target gene of PGC-1α that is associated with mitochondrial biogenesis [75]. Furthermore, a synergistic interaction between AMPK and SIRT1 has been shown to contribute to the regulation of gene expression associated with energy metabolism in the skeletal muscle of mice [76]. Consistent with this molecular mechanism, TMP supplementation significantly upregulated the mRNA levels of SIRT1 and Cyt c compared with the Con group ($p < 0.05$ or $p < 0.01$, Figure 7G,I). Under typical physiological circumstances, the phosphorylation of AMPK promotes SIRT1 activation, which subsequently activates several downstream proteins, including Cyt c and PGC-1α. This cascade enhances mitochondrial biogenesis and contributes to the alleviation of fatigue [10].

The ELISA results provided additional validation, demonstrating that TMP significantly enhanced AMPK and PGC-1α protein expression ($p < 0.05$, Figure 7J,K). When compared to the Con group, the high-dose TMP treatment group showed a marked increase in AMPK and PGC-1α levels, with average expression rising by 9.2% and 8.8%, respectively. The possible mechanism of the AMPK/PGC-1α signaling pathway regulating energy metabolism is illustrated in Figure 7L. Briefly, TMP supplementation upregulates the expression of AMPK, which in turn promotes the expression of SIRT1 and further enhances the PGC-1α-dependent transcriptional program. This process leads to increased expression of NRF1, TFAM, and Cyt c, thereby enhancing mitochondrial biogenesis and function, maintaining energy metabolism homeostasis, and ultimately ameliorating fatigue in mice. The results suggest that TMP participates in and regulates the AMPK/PCG-1α signaling pathway to promote mitochondrial biogenesis, thereby exerting anti-fatigue effects. These results are consistent with the observed outcomes related to ATP, Na^+-K^+-ATPase, and Ca^{2+}-Mg^{2+}-ATPase activities in muscle tissue.

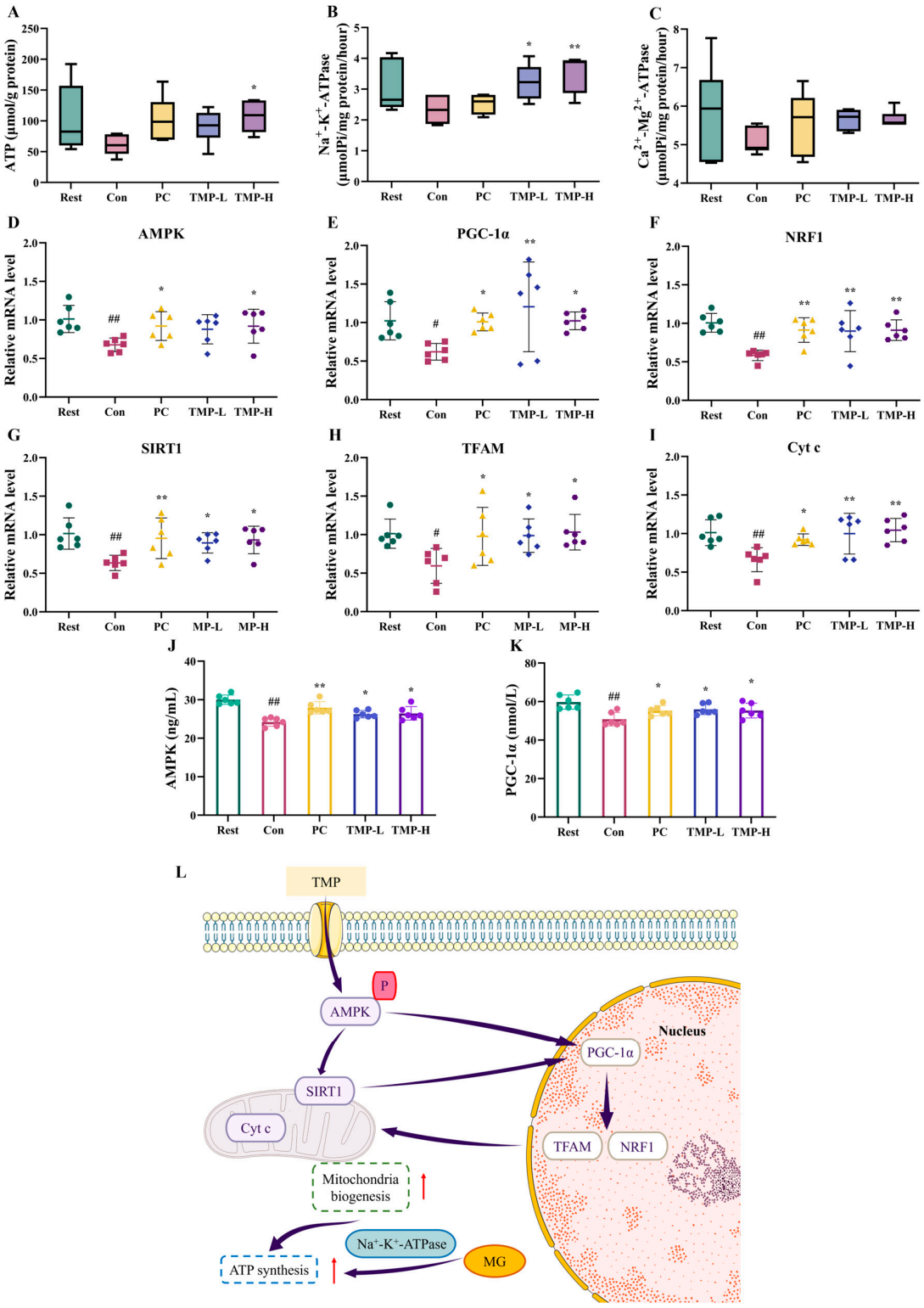

Figure 7. The contents of (**A**) ATP, (**B**) Na^+-K^+-ATPase, and (**C**) Ca^{2+}-Mg^{2+}-ATPase in mice muscle; the mRNA levels of (**D**) AMPK, (**E**) peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), (**F**) nuclear respiratory factor 1 (NRF1), (**G**) Sirtuin 1 (SIRT1), (**H**) mitochondrial transcription factor A (TFAM), and (**I**) Cytochrome C (Cyt c) and the proteins of (**J**) AMPK and (**K**) PGC-1α. (**L**) Schematic diagram of TMP's effects on the AMPK/PGC-1 pathway. (n = 6 in each group, # $p < 0.05$ and ## $p < 0.01$ indicated a significant difference vs. the Rest group, ** $p < 0.01$ indicates a significant difference vs. the model Con group, * $p < 0.05$ indicates a significant difference vs. the Con group.)

3.8. The Limitations of This Study

Despite these positive findings, the present study has several limitations. First, although TMP is mainly composed of low-molecular-weight peptides, which theoretically favor membrane permeability, direct experimental evidence regarding its gastrointestinal stability and intestinal absorption is lacking, and mechanistic interpretations derived from molecular docking should be regarded as hypothesis-generating. Second, 6-week-old mice differ from adult humans in muscle adaptation, digestive function, and recovery capacity, which may affect peptide absorption and in vivo efficacy. Therefore, although our results support the potential of TMP as a novel functional food ingredient for alleviating fatigue, further validation in human subjects is still needed to confirm its effectiveness and optimal dosage for human use.

4. Conclusions

This study obtained TMP from *Theragra chalcogramma* milt with 18.2% BCAA with 91.6% below 1000 Da. TMP, prepared by complex enzymatic hydrolysis of TM, exhibited a longer exhaustive swimming time in comparison to the Con group. Main biochemical indices demonstrated that TMP decreased the metabolite accumulations, reduced the level of oxidative stress, and increased the MG levels of mice. Additionally, TMP reduced the influx of LDH and CK from the muscle into the serum, and H&E results further revealed noticeable improvement in gastrocnemius muscle damage. Mechanistically, TMP promoted mitochondrial biosynthesis and enhanced exercise stamina, which was associated with upregulated expression of the AMPK/PGC-1α pathway. Therefore, TMP may be developed as a novel functional food with high anti-fatigue activity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu18050805/s1, Table S1. Specific primer sequences.

Author Contributions: J.Z.: data curation, methodology, formal analysis, writing—original draft. Y.D.: methodology, formal analysis, data curation. S.Z.: validation, software. T.Y.: writing—review & editing. C.F.: validation, data curation. X.Z.: writing—review & editing. H.H.: writing—review & editing, resources, funding acquisition, project administration. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All animal experiments conducted in this study were reviewed and approved by the Scientific Ethics Special Committee of the Academic Committee of Ocean University of China (Approval No. SPXY2023100830) on 8 October 2023 and were performed in compliance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Materials; further inquiries can be directed to the corresponding author.

Conflicts of Interest: Author X.Z. is affiliated with Kehai Jiantang Biology Co., Ltd., the provider of the *Theragra chalcogramma* milt (TM) materials, and there are no potential conflicts of interest. All other authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

TMP	*Theragra chalcogramma milt* peptides
TM	*Theragra chalcogramma* milt
Pap	Papain
Alk	Alkaline protease
Neu	Neutral protease
Fla	Flavor enzyme
Try	Trypsin
NR	Nitrogen recovery
DH	Degree of hydrolysis
CD	Circular dichroism spectroscopy
Rest	Resting control group
Con	Swimming control group
PC	Positive control group
TMP-H	High-dose group
TMP-L	Low-dose group
MDA	Malondialdehyde
GSH-Px	Glutathione peroxidase
SOD	Superoxide dismutase
BLA	Blood lactic acid
BUN	Blood urea nitrogen
LDH	Lactate dehydrogenase
CK	Creatine kinase
MG	Muscle glycogen
LG	Liver glycogen
H&E	Hematoxylin and eosin
ELISA	Enzyme-linked immunosorbent assay
AMPK	AMP-activated protein kinase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1α
NRF1	Nuclear respiratory factor 1
SIRT1	Sirtuin 1
TFAM	Mitochondrial transcription factor A
Cyt c	Cytochrome C

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