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Antifatigue Activities of Loach Protein Hydrolysates with Different Antioxidant Activities

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ABSTRACT: The antioxidant and antifatigue activities of two peptides of <5 kDa were determined, that is, loach peptide A (LPA, from a papain digestion) and loach peptide B (LPB, from a Flavorzyme digestion). Their degrees of hydrolysis were 21 ± 0.21 and 35 ± 0.32%, respectively. LPA fraction mainly possessed peptides of 1000 < MW < 3000 Da (65.41%), whereas LPB mainly possessed peptides of 500 < MW < 1000 Da (58.27%). LPA fraction contained 116.3 mg amino acid residues/g loach peptide powder of branched-chain amino acids, 1.42-fold that in LPB. LPA had stronger in vitro antioxidant activity than LPB. Compared with LPB, LPA increased swimming time more effectively and reduced blood urea nitrogen (BUN) and liver malonaldehyde (MDA) levels in mice, although both of them had significant antifatigue effects compared to the control (P < 0.05). Pearson correlation analysis showed that the antifatigue activity of loach peptide was highly correlated with its antioxidant activities.

KEYWORDS: *loach peptides, antioxidant activity, antifatigue activity, free radicals, amino acid composition*

INTRODUCTION

Fatigue is a complex phenomenon that can be described as a time-dependent exercise-induced reduction in the maximal force-generating capacity of a muscle.[1] It can cause various disorders of the bioregulatory, autonomic nervous, endocrine, and immune systems.[2] These disorders can lead to a reduction in exercise intensity or even to the interruption of activity.[3] Thus, the search for an antifatigue peptide would have potential commercial value.

There are several theories about the mechanisms of exercise-induced fatigue including the "exhaustion theory", the "clogging theory", the "radical theory", the "homeostasis disturbance theory", the "protective inhibition theory", and the "mutation theory".[4] Among these theories, the "radical theory" has been attracting more and more interest in recent years. It has been shown that intense exercise can produce an imbalance between the body's oxidant and antioxidant defense systems.[4,5] Free radicals will cause bodily injury by attacking molecules and cell organs when their production exceeds the body's clearance capability. High-intensity exercise can induce disturbance of pro-oxidant/antioxidant homeostasis. An imbalance between free radical production and clearance leads to an oxidative stress state, which may be involved in exercise-induced muscle damage or fatigue.[6] There has been considerable interest in how to inhibit free radicals to enhance physical ability.[2] The ability of molecules to scavenge free radicals might be a measure of their effect on oxidative stress.[7]

Compared with proteins, peptides as nutrients not only serve as an easily absorbed supplemental energy source but also promote the use of amino acids, proteins, and glucose. They have physiological activities that may alter the internal environment of the living body.[4,5] Some papers have indicated that peptides can provide immediate energy during exercise and are helpful during extensive exercise.[4,8] Other studies have shown that a number of peptides have antioxidant activities. For example, Yu et al.[2] found that a soybean peptide could significantly alleviate physical fatigue in mice. Ding et al.[5] found that a jellyfish collagen hydrolysate not only increased the climbing time of mice but also significantly reduced blood lactic and blood urea nitrogen (BUN) levels and increased hepatic and muscle glycogen. Moreover, Xue et al.[9] have found that rapeseed (*Brassica napus*) protein hydrolysate had stronger antioxidant activity toward peroxyl radicals and reducing power. However, few investigations have focused on the relationship between the antioxidant and antifatigue properties of peptides.

Therefore, in this study, two loach peptide fractions, loach peptide A (LPA) and loach peptide B (LPB), with different antioxidant activities were prepared. They were used to find the correlation between in vitro antioxidant activity and in vivo antifatigue activity of peptides.

MATERIALS AND METHODS

Loach Peptide Preparation. Live loach (*Misgurnus anguilliacaudatus*, 8.4 ± 1.1 g body weight and 9.8 ± 3 cm body length) were purchased from a local market in Guangzhou, Guangdong, China, and transported to the laboratory within 10 min. After killing, the meat (without head, tail, skin, bone, and blood) was collected and ground twice through a meat grinder with a 4 mm hole plate (MM12, Shaoguan Food Machine Co., Shaoguan, China). The ground meat was stored in a polyethylene bag at −18 °C until use, a maximum of 4 months. On the basis of our previous studies, two loach peptides with significantly different in vitro antioxidant activities were prepared, loach peptide A (LPA) (prepared from papain digestion) and loach peptide B (LPB) (prepared from Flavorzyme digestion). The LPA

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peptide fraction was prepared by starting with 50 g of loach meat mixed with 100 mL of distilled water and homogenized at a speed of 10000*g* for 1 min using a basic homogenizer (T25, Ika, Staufen, Germany). The homogenate was hydrolyzed with papain (6×10^5 U/g) (Baiao Biochemistry Co., Jiangmen, China) at 55 °C for 4.5 h. The enzyme to substrate ratio was 3:1000 (w/w). The hydrolysis was done at pH 7.0 with a pH-meter (SL1-PHS-3B pH-meter, Wuhan Midwest Instrument Co. Ltd., Wuhan, China) in a water bath shaker (New Brunswick Scientifics C24, Jintan, China). The LPB peptide fraction was similarly prepared. This time the homogenate was hydrolyzed with Flavorzyme (500 LAPU/g) (Novozyme Co., Danmark) at 50 °C for 20 h, again at pH 7.0 as previously described. The enzyme to substrate ratio was 4:1000 (w/w). After hydrolysis, both enzymes were inactivated by placing the hydrolysates in boiling water for 15 min. The hydrolysates were centrifuged in a GL-21 M refrigerated centrifuge (Xiangyi Instrument Co. Ltd., Changsha, China) at 5000*g* for 20 min, and the supernatants were fractionated using ultrafiltration membranes as part of a bioreactor system (Vivaflow 200, Vivascience, Sartorius, Goettingen, Germany) with a molecular weight cutoff (MWCO) of 5 kDa. The fraction with a molecular weight of <5 kDa was lyophilized (R2L-100KPS, Kyowa Vacuum Engineering, Tokyo, Japan) and stored in a desiccator for a maximum of 6 months for further use.

Animals. All of the in vivo tests were done by the School of Pharmaceutical Sciences of Southern Medical University (Guangzhou, China), who obtained university permission for all research protocols involving animal experiments; that is, they followed the guidelines of the ethics committee of Southern Medical University. Ninety male NIH mice (18−22 g, specific pathogen-free (SPF) grade, approval 2007A058) were purchased from the Academy of Experimental Animals Center (AEAC) of Southern Medical University. They were housed in an SPF level laboratory at the school. All experimental procedures were done with oversight and approval of the AEAC and were in strict accord with the *NIH Guide for the Care and Use of Laboratory Animals* (2002). Animals were allowed to adapt to their surroundings for 1 week before the experiments were begun. Mice were housed five or six per cage at room temperature (22 ± 2 °C) and moderate humidity (50 ± 10%) with a 12/12 h light/dark cycle; noise was <60 dB. They were fed a balanced murine diet provided by the AEAC and had drinking water available ad libitum. After adaptation, the 90 mice were randomly divided into 10 groups providing duplicate cages for each treatment: control, LPA treatment at high dose (LPA-H) and low dose (LPA-L), and LPB treatment at high dose (LPB-H) and low dose (LPB-L), with 9 mice each. One group from each set was used for the exhaustive swimming test. The other group was used for collecting blood to determine biochemical parameters related to fatigue after swimming for 30 min. For 4 weeks, the LPA-H and LPB-H mice were given 5 mg/(g day) of loach peptides LPA and LPB, respectively. The LPA-L and LPB-L mice were given 1 mg/(g day) of LPA and LPB, respectively. All of the loach peptides were given by intraperitoneal injection every day between 1:00 and 3:00 p.m. (The hydrolysates had some fishy taste and were not easily orally acceptable by the test animals, so the hydrolysates were given by intraperitoneal injection to ensure the tested doses.) The control groups received distilled water injections. After each daily treatment, all of the groups of mice were allowed to rest for 30 min and were then made to swim for 20 min to become accustomed to swimming (see below).

Determination of in Vitro Antioxidant Activities of Loach Peptides. *Hydroxyl Radical Scavenging Activity Assay.* The hydroxyl radical scavenging activity was assayed according to the method of Li et al.[10] with some modifications. A mixture of 600 μL of 1,10-phenanthroline (Sigma-Aldrich, St. Louis, MO, USA) (5.0 mM), 600 μL of $FeSO_4$ (5.0 mM,) and 600 μL of ethylenediaminetetraacetic acid (EDTA) (15 mM) was mixed with 400 μL of sodium phosphate buffer (0.2 M, pH 7.4). Then 600 μL of loach peptide (5−30 mg/mL) and 800 μL of H_2O_2 (0.01%) were added. The mixture was incubated at 37 °C for 60 min, and the absorbance was measured at 536 nm (UV754, Xianjian Scientific Instrument Co., Shanghai, China). The following equation was used to calculate activity:

$$\text{hydroxyl radical scavenging activity (\%)} = (A_S - A_0) \times 100/(A_c - A_0)$$

A_S is the absorbance of the sample, A_0 is the absorbance of the blank solution using distilled water instead of the sample, and A_c is the absorbance of a control solution in the absence of H_2O_2.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity Assay. DPPH radical scavenging activity was determined by using the method of Wu et al.[11] with a slight modification. Two milliliters of loach peptide (0.5−4.0 mg/mL) was mixed with 2.0 mL of 0.15 mM DPPH that was dissolved in 95% ethanol. The mixture was then shaken vigorously using a mixer (QT-1 Mixer, Tianchen Technological Co. Ltd., Shanghai, China) and kept for 30 min in the dark. The absorbance of the resulting solution was recorded at 517 nm. The scavenging activity was calculated using the following equation:

$$\text{scavenging activity (\%)} = (A_{\text{DPPH sample}} - A_{\text{sample control}}) \times 100/A_{\text{DPPH blank}}$$

$A_{\text{DPPH sample}}$ is the value for 2 mL of sample solution mixed with DPPH solution, $A_{\text{sample control}}$ is the value for 2 mL of sample solution mixed with 2 mL of 95% ethanol, and $A_{\text{DPPH blank}}$ is the value for 2 mL of 95% ethanol mixed with DPPH solution.

Cupric Ion Chelating Activity. The ability of loach peptides to chelate pro-oxidative Cu^{2+} was investigated using the method of Zhu et al.[12] In the chelation test, 1 mL of 2 mM $CuSO_4$ was mixed with 1 mL of pyridine (pH 7.0) and 20 μL of 0.1% pyrocatechol violet. After the addition of 1 mL of LPA or LPB (0.5−5.0 mg/mL), the disappearance of the blue color, due to dissociation of Cu^{2+}, was recorded by measuring the absorbance at 632 nm after 5 min of reaction. An equivalent volume of distilled water instead of the sample was used for the blank. The Cu^{2+} chelating activity of loach peptides was calculated as

$$Cu^{2+}\text{ chelating activity} = [(A_0 - A_s)/A_0] \times 100\%$$

where A_S is the absorbance of the sample and A_0 is the absorbance of the blank solution using distilled water instead of sample.

Measurement of Lipid Peroxidation Inhibition Activity in a Linoleic Acid Emulsion System. The lipid peroxidation inhibition activities of loach peptides were measured in a linoleic acid emulsion system according to the methods of Qian et al.[13] Briefly, 2.0 mL of LPA or LPB (5.0−20.0 mg/mL) was mixed with 2 mL of 2.5% linoleic acid dissolved in 95% ethanol. Then 4 mL of 50 mM sodium phosphate buffer (pH 7.0) and 2 mL of distilled water were added. The mixture was incubated in a 50 mL conical flask with a screw cap at 40 ± 1 °C in a dark room, and the degree of oxidation was evaluated by measuring the FeSCN value described below. The reaction solution (100 μL) incubated in the linoleic acid model system was mixed at different intervals during the incubation period with 9.7 mL of 75% ethanol, 0.1 mL of 30% NH_4SCN, and 0.1 mL of 20 mM $FeCl_2$ solution in 3.5% HCl. After 3 min of incubation, the SCN value was measured by the absorbance at 500 nm. An equivalent volume of distilled water instead of the sample was used for the blank.

$$\text{lipid peroxidation inhibition activity (\%)} = [1 - (A_{S,t=144\text{h}} - A_{S,t=0\text{h}})] \times 100/(A_{0,t=144\text{h}} - A_{0,t=0\text{h}})$$

$A_{S,\ t=144\ \text{h}}$ and $A_{S,\ t=0\ \text{h}}$ are the absorbances for the sample at 144 and 0 h, respectively; $A_{0,\ t=144\ \text{h}}$ and $A_{0,\ t=0\ \text{h}}$ are the absorbances for the blank at 144 and 0 h, respectively.

In Vivo Antifatigue Effect of Loach Peptides. *Exhaustive Swimming Test.* After the final treatment with LPA, LPB, or distilled water, the mice were allowed to rest for 30 min. They were then placed in the swimming tank (50 cm × 50 cm × 40 cm) with 30 cm deep water at 25 ± 1 °C. The current in the pool was generated by circulating water with a pump, and the strength of the current was adjusted to 8 L/min with a water flow meter (type F45500, Blue White Co., Westminster, CA, USA). The water was agitated to make the mice keep their limbs moving. The mice were determined to be exhausted

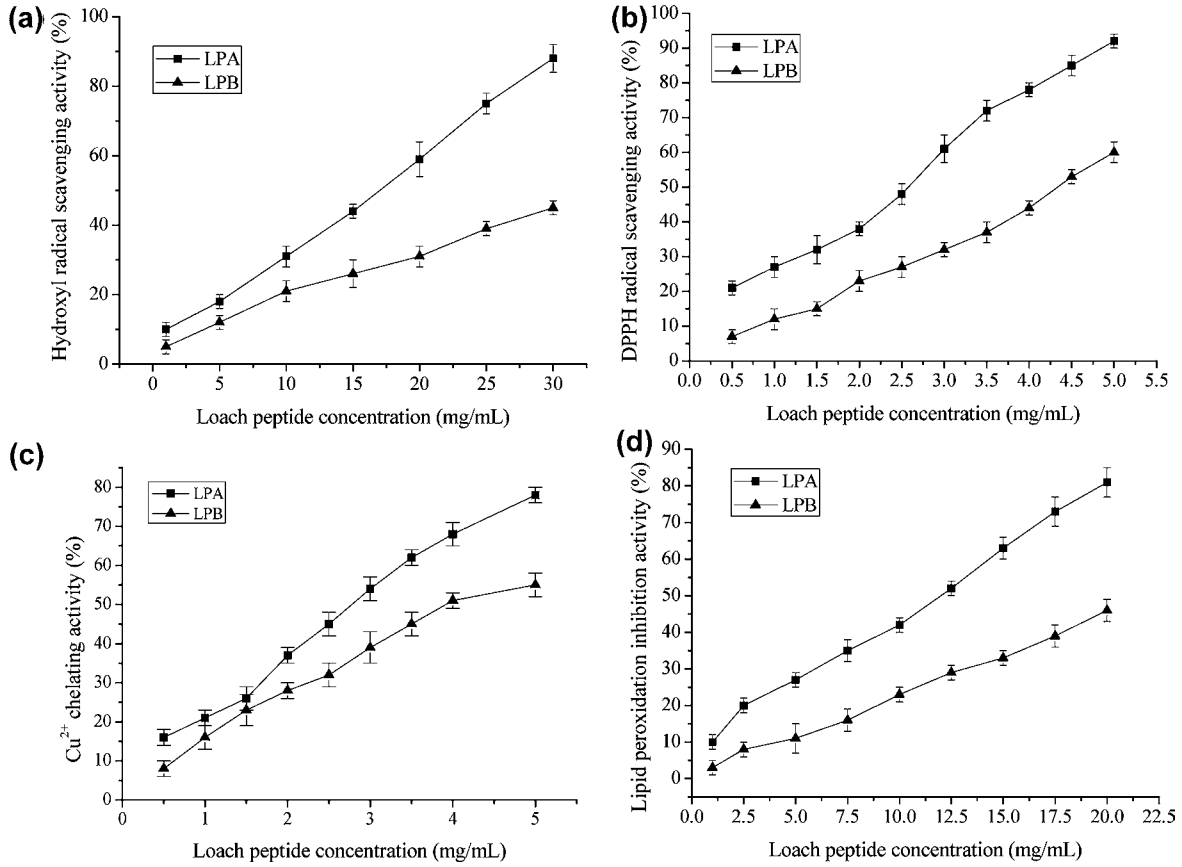

Figure 1. In vitro antioxidant activities of LPA and LPB: (a) hydroxyl scavenging activities of LPA and LPB; (b) DPPH radical scavenging activities of LPA and LPB; (c) Cu^{2+} chelating activities of LPA and LPB; (d) lipid peroxidation inhibition activities of LPA and LPB. The error bars represent standard deviation. Different letters indicate significant differences between groups ($P < 0.05$).

when they failed to rise to the surface to breathe after 7 s.[14] No mice drowned during these tests.

Measuring Biochemical Parameters Related to Fatigue. After the final treatment with LPA, LPB, or distilled water, the mice were allowed to rest for 30 min. Then they were placed in a swimming tank as described above. After swimming for 30 min, they were taken out and a blood sample was collected from the orbital sinus to determine the contents of lactate acid (LA), blood urea nitrogen (BUN), and malonaldehyde (MDA) and the activities of the antioxidative enzymes (superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT)).[14] The livers were taken to determine the content of liver glycogen (see below). The content of BUN was determined using a commercial diagnostic kit (product 000000280, Biosino Biotechnology and Science Inc., Beijing, China). LA was determined using a commercial diagnostic kit (product LC6351, Beijing Leadman Biochemistry Technology Co. Ltd., Beijing, China). The activities of SOD, GSH-Px, and CAT were determined using commercial kits from the Institute of Biological Engineering (IBE) of Nanjing Jianchen (Nanjing, China). The livers of the mice were dissected immediately after being taken out, washed with 0.9% saline, and blotted dry with filter papers. Liver samples (~100 mg) were accurately weighed, homogenized in 8 mL of the homogenization buffer from the IBE Liver Glycogen Assay Kit A043 (IBE of Nanjing Jianchen). The liver glycogen was determined according to the recommended procedures. The content of MDA was determined using IBE MDA Assay Kit A003-2 (IBE of Nanjing Jianchen).

Determination of the Degree of Hydrolysis, Molecular Weight Distribution, and Amino Acid Composition of LPA and LPB Fractions. The degree of hydrolysis is defined as the percentage of free amino groups cleaved from proteins, which was calculated from the ratio of α-amino nitrogen to total nitrogen. According to the method of Nilsang et al.,[15] the amino nitrogen content was determined by formaldehyde titration method. The total nitrogen content was determined according to the Kjeldahl method.[16]

Molecular weight distributions of LPA and LPB were determined according to the method of You et al.,[17] using gel permeation chromatography on a Superdex Peptide HR 10/300 GL (10 × 300 mm, Amersham Biosciences Co., Piscataway, NJ, USA) with UV detection at 214 and 280 nm.

The amino acid profiles of LPA and LPB were determined according to the method of You et al.[17] Amino acid composition was determined by high-performance liquid chromatography (Waters, Milford, MA, USA) equipped with a PICO.TAG column (3.9 × 300 mm, Waters). Total amino acids (except for tryptophan) were determined after hydrolysis at 110 °C for 24 h with 6 M HCl prior to derivatization with phenyl isothiocyanate. Alkaline hydrolysis was also done for determination of tryptophan level. The amino acid standards (Sigma-Aldrich) were done using the same conditions as for the samples.

Statistical Analysis. All of the tests were conducted in triplicate. The experimental data were expressed as the mean ± standard error. The results were subjected to one-way analysis of variance (ANOVA). Dunnett's T3 tests were performed to determine significant differences between samples within the 95% confidence interval using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSTION

In Vitro Antioxidant Activities of Loach Peptides. In biological systems, free radicals are generated in the form of reactive oxygen species (ROS), such as the superoxide anion and hydroxyl radicals, hydrogen peroxide, singlet oxygen, nitric oxide, and peroxynitrite. Overproduction of radicals will cause oxidative stress, which can lead to tissue damage and fatigue.[18]

Natural compounds with antioxidant activities can prevent the attack of free radicals. Many protein hydrolysates and their isolated peptides have shown promising in vitro antioxidant activity, including those from whey,[19] capelin,[20] and yellow stripe trevally.[21]

As shown in Figure 1, the in vitro antioxidant activities (including hydroxyl radical scavenging activity, DPPH radical scavenging activity, cupric ion chelating activity, and lipid peroxidation inhibitory activity) of LPA and LPB are concentration-dependent. When the concentration of loach peptide was increased, the antioxidant activity also increased. Moreover, LPA had stronger antioxidant activity than LPB at all concentrations used. When the concentration was 30 mg/mL, the hydroxyl radical scavenging activity of LPA was 88%, whereas that of LPB was 45% (Figure 1a). When the concentration of loach peptide was 4 mg/mL, the DPPH radical scavenging activity of LPA was 78%, 1.77-fold higher than that of LPB (Figure 1b). When the concentration of loach peptide was 5 mg/mL, the Cu^{2+} chelating activity of LPA was 78%, 1.42-fold higher than that of LPB (Figure 1c). When the concentration was 20 mg/mL, the lipid peroxidation inhibitory activity of LPA was 81%, 1.76-fold higher than that of LPB (Figure 1d). Above all, LPA had stronger in vitro antioxidant activity than LPB.

In Vivo Antifatigue Activities of Loach Peptides. *Exhaustive Swimming Time.* The forced swimming test, which is a commonly used technique for animal models of behavioral despair, has been used extensively for the evaluation of the antifatigue properties of novel compounds.[1,22] It can reflect the degree of fatigue by objectively assessing the physical ability of a body.[5,23] Both the high dose (5 mg/(g day)) and low dose (1 mg/(g day)) of LPA treatments significantly prolonged the swimming time of the mice ($P < 0.05$). As shown in Figure 2, the median exhaustion time of the LPA-H mice was

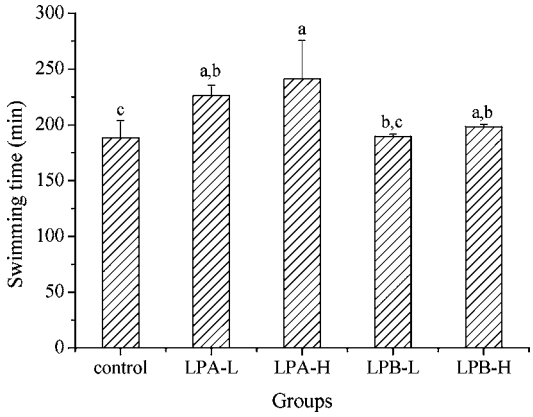

Figure 2. Effects of loach peptides on swimming time in mice. The error bars represent standard deviation. Different letters indicate significant differences between groups ($P < 0.05$).

241 min (28% greater than that of the water-injected control group), the median exhaustion time of the LPA-L mice was 226 min (20% greater than that of the control) ($P < 0.05$), indicating that LPA had an antifatigue effect. The median exhaustion time of the LPB-H mice was 209 min, which was 9 min longer than that of the LPB-L mice. The differences between LPA-H and LPB-H and between LPA-L and LPB-L were significant ($P < 0.05$). The results indicated that a peptide having strong antioxidant activity potentially might also have high antifatigue activity. During strenuous exercise, the sudden increase in muscle oxygen consumption and influx of oxygen causes a calcium overload in cells, leading to an influx of inflammatory cells into reperfused tissue.[24] Free radicals cause oxidative damage to nucleic acids, proteins, carbohydrates, and lipids, which would lead to fatigue. Antioxidants removing free radicals are key elements to combat fatigue and can be used in the treatment of several diseases, including chronic fatigue syndrome.[25] Thus, the antioxidant with higher activity can have stronger antifatigue activity.

To further confirm the antifatigue activities of LPA and LPB peptide fractions, some biochemical parameters, such as liver glycogen, LA, BUN, liver MDA contents, and SOD, CAT, and GSH-Px activities were determined in mice after they swam for 30 min.

Liver Glycogen. It is known that the endurance capacity of the body is markedly decreased if the energy is exhausted.[1,2] Energy for exercise is derived initially from the breakdown of glycogen. After strenuous exercise, muscle glycogen will be exhausted, and then energy will come from circulating glucose released by the liver.[26] Thus, the glycogen contents are sensitive parameters with a relationship to fatigue. As shown in Figure 3a, the contents of liver glycogen for all of the loach peptide treatment mice were significantly ($P < 0.05$) higher than that of control. The liver glycogen content of LPA-H was 21% higher than that of LPA-L ($P < 0.05$). The liver glycogen content of LPB-H was 13% higher than that of LPB-L ($P < 0.05$). Possible explanations may be that loach peptide may increase the content of liver and muscle glycogen of mice postexercise by improving glycogen reserves or by reducing the consumption of glycogen during exercise or both. Wang et al.[4] also found that a decapeptide (Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe) isolated from pig spleen delayed the consumption of liver glycogen and had antifatigue effects.

LA and BUN in the Blood. Blood lactic acid is the glycolysis product of carbohydrate under an anaerobic condition, and glycolysis is the main energy source for intense exercise in a short time. Therefore, blood lactic acid is one of the important indicators by which to judge the degree of fatigue.[5,27] Lactic acid will harm certain organs and produce fatigue.[4] If a substance can inhibit the accumulation of lactic acid and accelerate the clearance of lactic acid, it will show an antifatigue effect. As shown in Figure 3b, the contents of LA in LPA-H, LPB-L, and LPB-H treatment groups were significantly lower than that of the control ($P < 0.05$). The contents of LA at the high dose of loach peptide treatment were significantly lower ($P < 0.05$) than those of LA at the low dose.

BUN is the metabolism outcome of protein and amino acid. Urea is formed in the liver as the end product of protein metabolism and is carried by the blood to the kidneys for excretion.[5,27] Protein and amino acids have a strong catabolism when the body cannot obtain enough energy, and urea nitrogen will increase.[4] There is a positive correlation between urea nitrogen in vivo and exercise tolerance. The worse an animal is adapted to exercise, the more the urea nitrogen level increases.[28] As shown in Figure 3b, the BUN levels of mice were significantly lower by 17.5% in the LPA-H group compared to the control ($P < 0.05$). However, there was a decrease of 8.6% in the LPA-L group. The difference between LPA-H and LPA-L groups was significant ($P < 0.05$). The BUN level of the LPB-L group had no difference from that of the control ($P > 0.05$). The differences between LPA-H and LPB-H and between LPA-L and LPB-L were significant ($P < 0.05$).

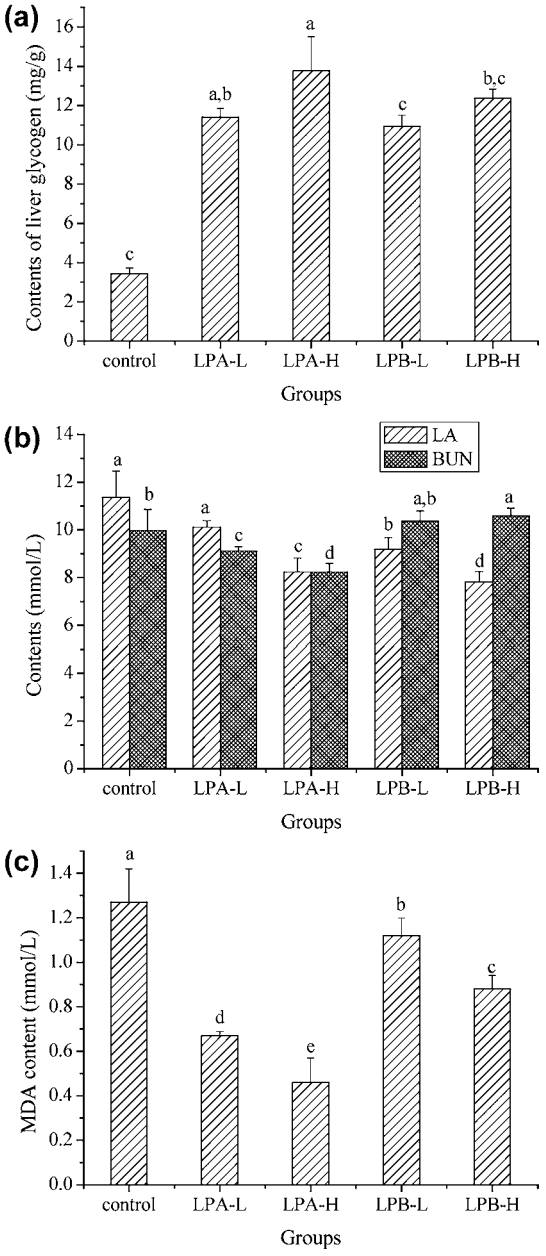

Figure 3. Effects of loach peptides on some biochemical parameters in mice: (a) content of liver glycogen of mice treated with loach peptides; (b) contents of LA and BUN in mice treated with loach peptides; (c) liver MDA level in mice treated with different loach peptides.

These results further indicated that peptide with strong antioxidant activity had a high antifatigue activity, which was consistent with the result of the exhaustive swimming time.

Liver MDA. Lipid peroxidation is an autocatalytic process, which is a common consequence of cell death. MDA is one of the end products in the lipid peroxidation process, and its levels are increased with aging.[5,29] As shown in Figure 3c, the liver MDA levels of all loach peptide treatment groups were significantly lower than that of the control ($P < 0.05$). Moreover, the MDA level showed a dose-dependent behavior. When the dose was increased, the MDA level was decreased. The higher antioxidant activity of the loach peptide treatment group produced a lower liver MDA level, indicating that high antioxidant activity of loach peptide had a close relationship with low injury to the body.

Antioxidative Enzymes in Mice. Growing evidence indicates that ROS are responsible for exercise-induced protein oxidation and contribute strongly to muscle fatigue.[30] Enzymatic antioxidant systems (including SOD, GSH-Px, and CAT) are important in scavenging free radicals and their metabolic products, as well as in maintaining normal cellular physiology, promotion of immunity, and prevention of various diseases.[31] As shown in Figure 4, the SOD level of the LPA-H group was

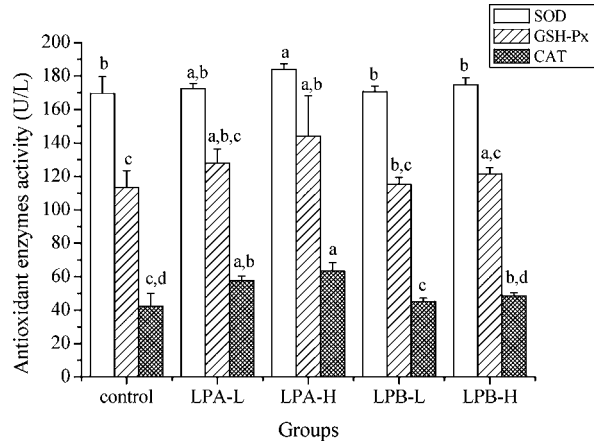

Figure 4. Effects of loach peptides on the activities of antioxidant enzymes in mice. The error bars represent standard deviation. Different letters indicate significant differences between groups within the same procedure ($P < 0.05$).

significantly higher than that of control and other groups ($P < 0.05$). However, the SOD activity of LPB groups (at both doses) was insignificantly different from that of the control ($P > 0.05$). Similarly, the activities of GSH-Px and CAT in the blood of the LPA-H group were significantly higher than that of the control. The activities of GSH-Px and CAT for both of the LPB-H and LPB-L groups were insignificantly different from those of the control. The SOD and CAT activities of the LPA treatment group were significantly higher than those of the LPB treatment group at a high dose ($P < 0.05$).

The results of in vivo antifatigue activity indicated that loach peptide with higher antioxidant activity had stronger antifatigue activity within the tested dose range. A strong antioxidant will help to scavenge excess free radicals during exercise. It will prevent the radicals from causing lipid peroxidation damage to cardiac and skeletal muscle during exercise-induced fatigue.[4]

Degrees of Hydrolysis, Molecular Weight Distributions, and Amino Acid Compositions of LPA and LPB Peptide Fractions. The antioxidant activities of loach protein hydrolysates were related to the degree of hydrolysis and enzymes used.[17] As shown in Table 1, the degrees of hydrolysis for LPA and LPB were 21 ± 0.21 and 35 ± 0.32%, respectively. The results were similar to those of our previous study[17] showing that loach protein hydrolysate at a degree of hydrolysis of 23%

Table 1. Degrees of Hydrolysis of LPA and LPB Peptide Fractions[a]

	LPA	LPB
degree of hydrolysis (%)	21 ± 0.21b	35 ± 0.32a

[a]Data with different lower case letters in a row are significantly ($P < 0.05$) different.

possessed much stronger antioxidant activities than that at a degree of hydrolysis of 33%.

The molecular weight (MW) distribution of LPA and LPB fractions is presented in Table 2. After ultrafiltration by a membrane of molecular weight cutoff of 5 kDa, LPA and LPB fractions were composed of >96% of peptides with MW < 5000 Da. The LPA fraction mainly possessed peptides of 1000 < MW < 3000 Da (65.41%), whereas the LPB fraction mainly possessed peptides of 500 < MW < 1000 Da (58.27%). Higher degree of hydrolysis and smaller molecular weight peptide fractions observed for LPB indicated that different degrees of hydrolysis and proteases led to different peptide chain lengths. This difference in peptide structures might explain the difference in the antioxidant and antifatigue activities.

Table 2. Molecular Weight (MW) Distributions of LPA and LPB Peptide Fractions (Percent)[a]

MW (Da)	LPA	LPB
MW > 5000	2.87 ± 0.76a	2.12 ± 0.57a
3000 < MW < 5000	9.11 ± 1.23a	5.83 ± 0.89b
1000 < MW < 3000	65.41 ± 2.24a	17.44 ± 3.11b
500 < MW < 1000	16.33 ± 1.48b	58.27 ± 2.58a
MW < 500	6.28 ± 1.04b	16.34 ± 1.87a

[a]Data with different lower case letters in a row are significantly ($P < 0.05$) different.

Amino acids in loach peptide are considered to be very important to their antioxidant activity. Several amino acids, such as tyrosine, methionine, histidine, lysine, and tryptophan, are generally accepted as antioxidants.[17] As shown in Table 3, the total contents of the above five antioxidant amino acids in LPA and LPB were 198 and 180.8 mg amino acid residues/g loach peptide powder, respectively. This might explain why the LPA peptide fraction possessed higher in vitro antioxidant activity than the LPB peptide fraction. Amino acids also play an important role in the regulatory metabolism involved in muscular activity. Some amino acids, especially the branched-chain amino acids, can improve exercise capability and markedly retard the catabolism of proteins in the muscle during exercise.[32] Branched-chain amino acids, including L-leucine, L-isoleucine, and L-valine, mainly act in the synthesis and metabolism of skeletal muscle. Branched-chain amino acids can effectively prevent the increase of brain (5-ht) ammonia content and resistance to central fatigue.[33] Moreover, branched-chain amino acids can reduce the movement of blood lactate accumulation, thus reducing the fatigue caused by the blood lactic acid.[34] As shown in Table 3, the content of branched-chain amino acids in the LPA peptide fraction was 116.3 mg amino acid residues/g loach peptide powder, 1.42-fold that in LPB. The results verified that supplementary exogenous branched-chain amino acids can reduce the blood lactic acid in rat and prevent fatigue.

Pearson Correlation Analysis. As shown in Table 4, Pearson correlation analysis revealed a significant association (negative or positive) among various indices for antioxidant activity and antifatigue activity. For all loach peptide-treated groups, swimming time, liver glycogen content, SOD activity, GSH-Px activity, and CAT activity exhibited positive correlation with the antioxidant activities (including hydroxyl radical scavenging activity, DPPH radical scavenging activity, Cu^{2+} chelating activity, and lipid peroxidation inhibition activity). On the other hand, LA content, BUN content, and MDA content showed negative correlation with the antioxidant activities.

Table 3. Amino Acid Compositions of Loach Peptides

	LPA		LPB	
amino acid	concentration[a] (mg amino acid residues/g loach peptide powder)	composition[b] (%)	concentration[c](mg amino acid residues/g loach peptide powder)	composition (%)
aspartic acid	69.7	8.74	81.3	10.10
glutamic acid	133	16.7	162	20.13
serine	28.1	3.52	37.5	4.66
glycine	46.9	5.88	52.1	6.47
histidine	31.1	3.9	26.8	3.33
arginine	35.4	4.43	37.3	4.63
threonine	37.7	4.72	38.4	4.77
alanine	55.9	7.01	53.2	6.61
proline	43.7	5.48	41.2	5.12
tyrosine	27.9	3.5	25.6	3.18
valine	29.4	3.69	22.8	2.83
methionine	19.6	2.45	18.5	2.30
cystine	0.2	0.02	0.3	0.04
isoleucine	31	3.88	25	3.11
leucine	55.9	7.01	34	4.22
tryptophan	65.7	8.23	62.1	7.72
phenylalanine	33	4.14	39	4.85
lysine	53.7	6.73	47.8	5.94
total	798	100	804.9	100.00

[a]One gram of LPA fraction from the 0−5 kDa fraction contained 850 mg of total estimated protein. Thus, the approximate yield of identified amino acid residues (amino acids minus H_2O) accounted for 798/850 or approximately 94% of the nominal protein. [b]Normalized so that the observed amino acid residues add up to 100% of the total amino acid residues. [c]One gram of LPB fraction from the 0−5 kDa fraction contained 855 mg of total estimated protein. Thus, the approximate yield of identified amino acid residues (amino acids minus H_2O) accounted for 804.9/855 or approximately 94% of the nominal protein.

Table 4. Pearson Correlation Analysis between Antifatigue and Antioxidant Activities[a]

	hydroxyl radical scavenging activity		DPPH radical scavenging activity		Cu^{2+} chelating activity		lipid peroxidation inhibition activity	
	LPA	LPB	LPA	LPB	LPA	LPB	LPA	LPB
swimming time	0.494	0.997**	0.386	0.967**	0.388	0.963**	0.489	0.969**
liver glycogen	0.823**	0.994**	0.781	0.908*	0.783	0.904*	0.845*	0.974**
LA content	−0.812*	−0.587	−0.908*	−0.821*	−0.908*	−0.825*	−0.848*	−0.49
BUN content	−0.765	0.696	−0.859*	0.425	−0.86*	0.419	−0.788	0.739
MDA content	−0.676	−0.635	−0.827*	−0.853*	−0.826*	−0.856*	−0.755	−0.547
SOD activity	0.841*	0.868*	0.934**	0.659	0.932**	0.648	0.972**	0.918**
GSH-Px activity	0.588	0.939**	0.524	0.776	0.524	0.77	0.62	0.949**
CAT activity	0.691	0.929**	0.706	0.757	0.705	0.749	0.788	0.955**

[a]*, significant at $P < 0.05$; **, significant at $P < 0.01$.

Swimming time was highly correlated with the antioxidant activity for LPB. The Pearson correlation coefficients between swimming time of the LPB-treated group and hydroxyl radical scavenging activity, DPPH radical scavenging activity, Cu^{2+} chelating activity, and lipid peroxidation inhibition activity were 0.997, 0.967, 0.963, and 0.969, respectively ($P < 0.01$). Similarly, liver glycogen content was highly correlated with the antioxidant activity for LPB. The Pearson correlation coefficients were 0.994, 0.908, 0.904, and 0.974, respectively ($P < 0.05$). However, for LPA-treated groups, the Pearson correlation coefficients of swimming time and liver glycogen content and its antioxidant activities were not significant, except for those for liver glycogen content versus hydroxyl radical scavenging activity ($r = 0.823$, $P < 0.01$) and lipid peroxidation inhibition activity ($r = 0.845$, $P < 0.05$). For LPB-treated groups, the antioxidant enzyme activities in vivo (including SOD activity, GSH-Px activity, and CAT activity) were highly correlated with its hydroxyl radical scavenging activity and lipid peroxidation inhibition activity ($P < 0.05$). The LA contents of LPA treatment groups were negatively correlated with the antioxidant activities. The Pearson correlation coefficients between swimming time of the LPB-treated group and hydroxyl radical scavenging activity, DPPH radical scavenging activity, Cu^{2+} chelating activity, and lipid peroxidation inhibition activity were −0.812, −0.908, −0.908, and −0.848, respectively ($P < 0.05$). Above all, Pearson correlation analysis showed that the antifatigue activity of loach peptide was highly correlated with its antioxidant activities.

Conclusions. Although there are a few studies on the antioxidant or antifatigue activities of hydrolysates from food protein, no paper has been found to determine if the hydrolysates have any relationship with their antioxidant activities. Therefore, this study was performed to find the answer for the first time. The results indicated that the LPA fraction (with a degree of hydrolysis of 21 ± 0.21%) mainly possessed peptides of 1000 < MW < 3000 Da (65.41%), whereas LPB (with a degree of hydrolysis of 35 ± 0.32%) mainly possessed peptides of 500 < MW < 1000 Da (58.27%). The LPA fraction contained 116.3 mg amino acid residues/g loach peptide powder of branched-chain amino acids, 1.42-fold that in LPB. LPA had stronger antioxidant and antifatigue activities than LPB. Pearson correlation analysis showed that the antifatigue activity of loach peptide was highly correlated with its antioxidant activities. This indicated that the antifatigue activity was highly related with the antioxidant activity. The results would be meaningful in developing antifatigue functional foods by evaluating their in vitro antioxidant activities as a primary screening.

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Notes

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