Antioxidant activity and anti-exercise-fatigue effect of highly denatured soybean meal hydrolysate prepared using neutrase

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Abstract Highly denatured soybean meal is a by-product of soybean oil extraction obtained through high-temperature desolventization. High-temperature treatment can result in soybean protein denaturation. Compare with ordinary soybean meal, the protein structure of highly denatured soybean meal has changed. Highly denatured soybean meal was pretreated with thermal treatment or ultrasonication, and then hydrolyzed with neutrase. The ultrasonicated hydrolysate exhibited better antioxidant activity than the thermally treated hydrolysate. The ultrasonication increased 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity by 8.31 % and reduction capacity by 10.19 %. The highly denatured soybean meal hydrolysate ultrasonicated at 400 W exhibited the highest antioxidant activity. The DPPH radical scavenging activity was 56.22 % and reduction capacity was 0.717. The ultrasonicated hydrolysate at 400 W was fractionated using ultrafiltration into three fractions: I (>10 kDa), II (5 kDa to 10 kDa), and III (<5 kDa). The *in vitro* antioxidant activity and others *in vivo* anti-exercise-fatigue effect of the three fractions (I, II, and III) were determined. Fraction III exhibited the highest DPPH radical scavenging activity and reduction capacity, improved the hemoglobin and hepatic glycogen content and reduced blood urea nitrogen and blood lactic acid. Fraction III improved the activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) and reduced the malonaldehyde (MDA) content in mouse livers. Therefore, the highly denatured soybean meal hydrolysate has an anti-oxidative effect and it significantly alleviates exercise-fatigue in mice. Amino acids of hydrolysate were determined.

Results showed that the antioxidant activity and anti-exercise-fatigue effect were related to the amino acid compositions.

Keywords Highly denatured soybean meal · Physical modification · Antioxidant activity · Anti-exercise-fatigue effect

Introduction

Fatigue is a physiologic condition that occurs after physical or mental exertion. Fatigue indicates a temporary decline in the ability to work or it could be a symptom of disease. Fatigue can be classified into exercise fatigue and chronic fatigue. Exercise fatigue is a decline in physical function or exercise capacity caused by a certain amount of movement. Chronic fatigue is not mitigated by rest accompanied by sore throat, headache, muscle and joint pain, and memory loss, and it may occur repeatedly and last for more than six months (Ream and Richardson 1997; Chaudhuri and Behan 2004; Tanaka et al. 2008). Considering fatigue is related to many research methods, the mechanism of fatigue is still controversial. Currently, the major theories regarding the mechanism of exercise fatigue include clogging theory, exhaustion theory, homeostasis disturbance theory, radical theory, protective inhibition theory, disorder of metabolic ion theory, and mutation theory (Harman 1956; Pedersen et al. 2004; Sugino et al. 2007; Wang et al. 2008; You et al. 2011).

Among these assumptions, the radical theory has attracted widespread interest. Exercise induces the production of excess free radicals, which damage organelles such as the mitochondria and the cell membrane, causing a series of cellular metabolic disorders, a decline in working capacity and fatigue (Wang et al. 2008). The *in vivo* antioxidant enzyme system, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and catalase (CAT), remove free radicals and their

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metabolites to maintain physiologic cellular activity and are resistant to exercise-induced oxidative damage (Yu et al. 2006). Studies showed that exogenous dietary antioxidants remove excessive free radicals and reduce exercise-induced oxidative injuries, thereby alleviating exercise fatigue. This ability is likely because exogenous antioxidants inhibit the destruction of liposomal membranes and reduce erythrocyte hemolysis (Mizuno et al. 2008; Ding et al. 2011; Choi et al. 2012). However, the underlying mechanism is still unclear. Nevertheless, consumers aim to protect themselves from fatigue through exogenous natural antioxidant supplements to alleviate and to resist various diseases caused by aging and free radicals (You et al. 2011).

Soybean peptides scavenge free radicals, inhibit lipid peroxidation, and provide energy immediately during exercise, as well as high digestion and absorption rates (Pena-Ramos and Xiong 2002; Korhonen and Pihlanto 2003; Friedman and Brandon 2001). Soybean peptides are usually prepared through enzyme-hydrolyzed soybean protein isolates (Beermann et al. 2009). Highly denatured soybean meal is a by-product of soybean oil extraction. It is obtained through high-temperature desolventization for 15 min at 100 °C. Part of the anti-nutritional elements is removed after high temperature treatment. And high temperature treatment can result in soybean protein denaturation. The soybean protein structure of highly denatured soybean meal has changed. The highly denatured protein is obtained through high-temperature desolventization. Highly denatured soybean meal contains about 43 % protein. The highly denatured soybean meal is difficult to breakdown enzymatically; thus, it is often used as feed. Only small amounts of highly denatured soybean meal is used for production of fermented foods, resulting to a huge waste of high-quality proteins and physiologically active substances (Wang and Johnson 2001). Physical modification changes the structure of proteins, thereby improving the efficiency of enzymolysis and improving their functional properties (Considine et al. 2007; Liu and Zhao 2010). Wu et al. (2010) reported the antioxidant effect of soybean isolate protein enzymatic hydrolysate treated by ultrasonic. The results indicated that the ultrasonic pretreatment could promote the antioxidant activity of soybean protein hydrolysate. Jia et al. (2010) studied the effects of ultrasonic treatment on kinetic characterisation and ACE-inhibitory activity during the hydrolysis of defatted wheat germ protein. The results indicated that ultrasonic treatment during proteolysis could facilitate the enzymatic hydrolysis of defatted wheat germ protein, whereas ultrasonic pretreatment could promote the release of ACE-inhibitory peptides from defatted wheat germ protein during enzymatic hydrolysis. Antioxidant and anti-fatigue peptides are obtained from the physical modification of highly denatured soybean meal, which is cost-effective and safe method for obtaining a rich supply of raw materials and easily accepted by consumers (Wang et al. 2006; Nina and Liisa 2007).

We investigated the effects of thermal pretreatment and ultrasonic pretreatment on the antioxidant activity of highly denatured soybean meal hydrolysate. The *in vitro* antioxidant activity and *in vivo* anti-exercise-fatigue effect of highly denatured soybean meal hydrolysate with different molecular weights (>10 kDa, 5 kDa to 10 kDa, and <5 kDa) were determined. The relationship between the antioxidant and the anti-exercise-fatigue properties of soybean peptides was studied.

Materials and methods

Materials

Highly denatured soybean meal (43 % protein) was obtained from Heilongjiang Jiusan Oil & Fat Co. (Harbin, China). Neutrase was purchased from Novo Nordisk (Bagsvaerd, Denmark). 1,1-diphenyl-2-picryl hydrazyl (DPPH) was purchased from Sigma Chemical Co. (St. Louis, MO). All the kits including blood urea nitrogen (BUN), hepatic glycogen and muscle glycogen, blood lactic acid, hemoglobin (Hb), malonaldehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and bradford protein were purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). White mice were obtained from Hanfang Experimental Animal Breeding Institute (Harbin, China). All other chemicals used in the experiments were of analytical grade.

Thermal treatment of highly denatured soybean meal

The highly denatured soybean meal was dispersed in distilled water at a protein concentration of 40 mg/ml. It was kept in a water bath then heated from 20 °C to a specific temperature (70, 80, 90, and 100 °C) at 10 °C/min, maintained 30 min at the specific temperature then cooled down to 45 °C for hydrolysis.

Ultrasonic treatment of highly denatured soybean meal

The highly denatured soybean meal was dispersed in distilled water at a protein concentration of 40 mg/ml. It was treated using an ultrasonic cleaning machine (KQ-500DV, Ultrasonic instruments Co., Kunshan, China) at 45 °C at different levels of power output (0, 200, 300, 400, and 500 W) for 30 min, and then was hydrolyzed in a water bath at 45 °C.

Enzymatic hydrolysis

The highly denatured soybean meal was dispersed in distilled water at a protein concentration of 40 mg/ml, and then pretreated with thermal treatment or ultrasonic treatment.

After treatment, the treated protein solution was adjusted to pH 7.5 with 1 M NaOH, and then incubated in a water bath at 45 °C. Neutrase was added at an enzyme-to-substrate ratio of 32 000 U/g protein. The mixtures of protein and enzyme were incubated at 45 °C for 4 h. The pH was maintained at 7.5 by adding 1 M NaOH during the enzymatic reaction. After hydrolysis, the temperature was increased to 100 °C and maintained for 10 min to eliminate the enzyme. The pH of the protein and enzyme solution was adjusted to 4.3 with 1 M HCl. The protein and enzyme solution was centrifuged at 4000 g for 15 min at 20 °C. The supernatants were freeze-dried (FDU-1100, Freeze-Dryer, Tokyo, Japan), and the lyophilized hydrolysates were stored at 4 °C. The control was the hydrolysate without thermal and ultrasonic treatment.

Radical scavenging activity on DPPH free radical

DPPH (1,1-diphenyl-2-picryl hydrazyl) radical scavenging activity was measured by the method of Saiga et al. (2003). Highly denatured soybean meal hydrolysate was dispersed in distilled water at 40 mg/ml. Up to 1.00 ml of hydrolysate was mixed thoroughly with 4.00 ml of DPPH (100 µmol/l). The solution was protected from light for 30 min. The absorbency of the mixture was measured at 517 nm, and denoted as A_i , and 95 % ethanol was used for normalization. Similar to the previous operation, 1.00 ml of 95 % ethanol was thoroughly mixed with 4.00 ml of DPPH (100 µmol/l). The absorbency was measured and denoted as A_e . Finally, 1.00 ml of hydrolysate was thoroughly mixed with 4.00 ml of 95 % ethanol. The absorbency was measured and denoted as A_j . The DPPH free radical scavenging rate was calculated in accordance with the following formula:

$$\text{DPPH radical scavenging activity (\%)} = [1 - (A_i - A_j) / A_e] \times 100$$

Determination of reduction capacity

Reduction capacity was measured by the method of Liu et al. (2009). Highly denatured soybean meal hydrolysate was dispersed in distilled water at 40 mg/ml. Up to 2.50 ml of 1 % $K_3[Fe(CN)_6]$ and 2.50 ml of phosphate buffer (pH 6.6) was added to 1.00 ml of hydrolysate, shaken well, and heated at 50 °C for 20 min. Then, 2.50 ml of trichloroacetic acid (10 %) was added to the solution. The mixture solution was centrifuged for 10 min at 3 000 r/min. The precipitate was removed by centrifugation.

The supernatant was retained. Up to 2.50 ml of H_2O and 0.50 ml of $FeCl_3$ (0.1 %) was added to 2.50 ml of the supernatant. The mixed solution was shaken well. And the absorbency of the mixed solution was measured at 700 nm, and denoted as A. The absorbency is related to reduction

capacity. The absorbency is higher, the reduction capacities will be stronger. The 30 µg/ml Vc was used as a control. The reduction capacities of Vc and the highly denatured soybean meal hydrolysate were compared.

Isolation of soybean peptides

Two polyethersulfone membranes that can separately retain substances with relative molecular masses of 10 and 5 kDa were used. During ultrafiltration (Amicon Stirred Cell 8400, Millipore, USA), the highly denatured soybean meal hydrolysates were passed through these membranes. The filtrate from the ultrafiltration membrane with a molecular mass cut-off of 10 kDa was collected and used as the initial solution for ultrafiltration using the membrane with a molecular mass cut-off of 5 kDa at 20 °C. The front and back pressures of the membrane were 1 and 0.4 MPa, respectively. Subsequently, the external and internal solutions were collected. Three fractions with relative molecular masses of I (>10 kDa), II (5 kDa to 10 kDa), and III (<5 kDa) were obtained. Three fractions recovered were lyophilized in a freeze-drier (Tokyo).

Anti-exercise-fatigue experiment

A total of 40 mice weighing around 18 g were randomly assigned into four groups: Group A (control group), Group B (perfused with the >10 kDa hydrolysate), Group C (perfused with the 5 kDa to 10 kDa hydrolysate), and Group D (perfused with the <5 kDa hydrolysate). Each group included 10 mice. The gastric perfusion dosage of each hydrolysate group was 5 mg/g body weight day, and the control group was administered with the same volume of distilled water. The mice in each group were fed at 20 °C to 25 °C with a daylight time of 12 h. The mice were continuously administered with the test substance for 30 days.

The mice were normally fed and timely perfused. The bodies were weighed before administration and 10, 20, and 30 days after administration. The average weight of each group was calculated.

Indicators were measured after 30 days of continuous administration. The mice were forced to swim for 30 min in a warm water bath at 30 °C after the last administration. The mice were removed, patted dry, and allowed to rest for 60 min. Blood was collected through the eyeball and blood urea nitrogen was determined in accordance with the instructions of the kit. Blood lactic acid and hemoglobin were also determined in accordance with the instructions of the kit. The livers and quadriceps of the mice were collected and hepatic glycogen and muscle glycogen were determined in accordance with the instructions of the kit. The experimental procedure was an experiment. Three replications of the whole experiments described in anti-exercise-fatigue experiment were conducted.

In vivo antioxidant activities experiment

A total of 40 mice, weighing around 18 g each, were randomly assigned into two groups: Group M (control group) and Group N (soybean peptide group). Highly denatured soybean meal hydrolysate with a relative molecular mass of less than 5 kDa were intragastrically administered to group N at 5 mg/g body weight day. The same volume of distilled water was intragastrically administered to group M. All mice were fed at 20 °C to 25 °C for 30 days of continuous administration. Then, 10 mice from group M were assigned to group M1 (control static group) and 10 mice from group N were assigned to group N1 (soybean peptide static group). The mice in group M1 and group N1 were killed by cervical dislocation 30 min after the last administration. Their livers were immediately removed to determine the SOD and GSH-Px activity and the MDA content in accordance with the instructions of the kit. Then, 10 mice from the remaining mice in group M and group N and were assigned to group M2 (control exercise group) and N2 (soybean peptide exercise group), respectively. The mice were forced to swim for 30 min in a warm water bath at 30 °C after the last administration. The mice were removed from the water and patted dry. The mice were killed and their livers were immediately collected to measure the SOD and GSH-Px activity and the MDA content in accordance with the instructions of the kit. The process of in vivo antioxidant activities experiment was presented in Fig. 1. The experimental procedure was an experiment. Three replications of the whole experiments described in Figure 1 were conducted.

Determination of amino acids

The total amino acid compositions of the hydrolysates with different molecular weight of I (>10 kDa), II (5 kDa to 10 kDa), and III (<5 kDa) were determined after acid hydrolysis. The hydrolysate samples were treated with chloroform–methanol (2:1, v/v) mixed solution in an ampoule and then added with 6 M HCl. After sealing the ampoule, the hydrolysates were hydrolyzed at 110 °C for 24 h under a nitrogen atmosphere. After the hydrolysates were evaporated to dryness, they were dissolved in 0.02 M HCl in vacuum for 30 min, filtered, and then loaded on a Model L-8800 amino acid analyzer (Hitachi Limited, Japan) for amino acid analysis. The experimental procedure was an experiment. Three replications of the whole experiments were conducted.

Statistical analysis

All experiments were done in triplicate. The results were expressed as means±SD and analyzed using the SPSS 13.0 software package. Intergroup differences were analyzed using

ANOVA. Differences were considered significant at $p < 0.05$ and very significant at $P < 0.01$.

Results and discussion

Effects of thermal treatment on antioxidant activity of highly denatured soybean meal hydrolysate

Effects of thermal treatment on DPPH radical scavenging activity and reduction capacity of highly denatured soybean meal hydrolysate were shown in Fig. 2. The radical scavenging activity and reduction capacity were enhanced with increasing temperature. The antioxidant activity of hydrolysate was the strongest at 90 °C. The highest DPPH radical scavenging activity was 56.22 % and the highest reduction capacity was 0.717. At 100 °C, the DPPH radical scavenging activity slightly decreased ($p > 0.05$), and the reduction capacity decreased significantly ($p < 0.05$). Thermal treatment changed the protein structure and facilitated contact between the enzyme and the protein, thereby improving the antioxidant capacity of the hydrolysate (La et al. 2002). However, excessive heating, such as at 100 °C for 30 min, caused the protein to unfold and exposed a large number of hydrophobic groups (Cui et al. 2008; Liu and Zhao 2010). The hydrophobic interactions caused by polymerization of the protein buried the active sites inside the molecule and hindered proteolysis, which decreased the antioxidant activity at 100 °C (Costaa et al. 2007).

Effects of ultrasonic treatment on antioxidant activity of highly denatured soybean meal hydrolysate

The effects of ultrasonic treatment on DPPH radical scavenging activity and reduction capacity of highly denatured soybean meal hydrolysate were shown in Fig. 3. Increasing ultrasonic power enhanced the radical scavenging capacity and reduction capacity of the hydrolysate, which may be caused by the cavitation effect of ultrasonic waves (Song et al. 2008; Jia et al. 2010). The bubbles that formed because of cavitation fracture and instantly generate strong shear forces. The shearing changes the structure of proteins and exposes certain groups, which improves contact with enzymes and the antioxidant activity of the hydrolysate (Considine et al. 2007). Compare with the thermal treatment, the ultrasonication increased the DPPH radical scavenging activity of highly denatured soybean meal hydrolysate by 8.31 % and its reduction capacity by 10.19 %. Ultrasonication resulted in better performance than thermal treatment. The reduction capacities of Vc was measured. The reduction capacities was 0.252. Compare with the reduction capacities of Vc, the reduction capacities of highly denatured soybean meal hydrolysate was higher. The result showed that the highly denatured soybean meal hydrolysate had a antioxidant activity, and

Fig. 1 Process of in vivo antioxidant activities experiment. Details were described in the [Methods and materials](#) section. Three replications of the whole experiments described in Fig. 1 were conducted

provided some basis study in selection the natural antioxidant agents (Chen et al. 2013). Considering the antioxidant activity only increased slightly when the intensity of ultrasonication

was increased from 400 W to 500 W ($p > 0.05$), the hydrolysate ultrasonicated at 400 W was used for all subsequent experiments.

Fig. 2 Effect of thermal treatment on DPPH radical scavenging activity and reduction capacity of highly denatured soybean meal hydrolysate. Means with different letters (a–d) differ significantly ($p < 0.05$)

Fig. 3 Effect of ultrasonic treatment on DPPH radical scavenging activity and reduction capacity of highly denatured soybean meal hydrolysate. Means with different letters (a–d) differ significantly ($p < 0.05$)

Isolation of soybean peptides

The DPPH radical scavenging activity and reduction capacity of three ultrafiltration fractions with different molecular weights (I (>10 kDa), II (5–10 kDa), and III (<5 kDa)) were shown in Table 1. Among the three fractions, III fraction exhibited the highest antioxidant activity ($p < 0.05$). Guo et al. (2009) noted that the low molecular weight fraction (< 1 kDa) from the hydrolysate royal jelly protein had the highest peroxidation inhibition rate. Liu et al. (2010) reported that the fraction (< 3 kDa) from the hydrolysate of porcine plasma protein had the greatest reduction capacity and DPPH radical scavenging activity. The result showed that the fraction with small molecular weights had highly antioxidant activity.

Effects of anti-exercise-fatigue

All mice in each group were vivacious, with good mental status and smooth hair. No adverse reaction was observed. Table 2 demonstrates the effect of soybean peptides on the body weight of mice. Compared with the control group, no significant differences in body weight were found in all administration groups during the entire experimental period ($p > 0.05$). This

finding suggests that the soybean peptides had no obvious effect on the body weight of mice (Yamamoto et al. 2003).

Movement requires large amounts of oxygen. Insufficient oxygen causes hypoxia and inhibits aerobic metabolism, which undermines the exercise capacity. The large amount of hemoglobin in erythrocytes acts as buffer and carries carbon dioxide and oxygen; thus, the hemoglobin content affects the exercise capacity (Xu and Luo 2001; Nozaki et al. 2009). The hemoglobin content in mice was measured after 30 days of intragastric administration. The hemoglobin content of the mice in group B were significantly higher than those in group A ($p < 0.05$). Group C and group D had significantly higher hemoglobin content than group A ($P < 0.01$; Fig. 4). The results indicate that all of the hydrolysates with different molecular weights increased hemoglobin content and mitigated the hypoxic conditions caused by prolonged exercise and improved aerobic metabolism.

During exercise, the proteins in muscles are broken down to provide energy when energy from fat and sugar metabolism is insufficient. This catabolic reaction destroys structural components and affects normal biological functions and muscle contraction. Consequently, the body may present with fatigue

Table 1 DPPH radical scavenging activity and reduction capacity of peptide fractions with different molecule weight

	Fraction I	Fraction II	Fraction III
DPPH (%)	40.52±1.06 ^c	54.21±1.14 ^b	65.33±1.18 ^a
Reduction capacity (A)	0.552±0.014 ^c	0.732±0.019 ^b	0.846±0.015 ^a

All fractions: 40 mg/ml

^{a–c} Means in the same row with different superscript letters differ significantly ($p < 0.05$)

Table 2 Comparison of mice weight

Group	Original body weight (g)	After 10 d (g)	After 20 d (g)	After 30 d (g)
Group A	18.18±1.13	21.27±0.97	23.92±1.52	28.36±1.86
Group B	18.22±1.09	21.78±1.01	24.77±1.26	27.78±1.75
Group C	18.06±1.08	21.18±1.14	24.09±1.58	28.12±1.05
Group D	18.46±0.89	20.95±0.93	24.86±1.04	27.93±2.07

Data express the mean±SD, $n=3$ in each group. No significant differences in body weight were found in all administration groups d at the same period of time

Fig. 4 Effect of hydrolysate on Hb of the mice. Data express the mean $\pm$ SD, $n=3$ in each group, * $p<0.05$, ** $P<0.01$, compared with group A (control group)

and other symptoms (Koo et al. 2004). The in vivo blood urea nitrogen increases after prolonged time exercise. Blood urea nitrogen is an indicator of protein breakdown and damage and recovery of muscle cells. Therefore, blood urea nitrogen can be used to assess the physical loading capacity during exercise (Wang et al. 2006; Zhang et al. 2006). The blood urea nitrogen of mice was measured after swimming. The results were shown in Fig. 5. The blood urea nitrogen of all mice from the intragastric administration groups were lower than the control group, which significantly decreased 24.97 % ($P<0.01$), 17.93 % and 16.34 % ($p<0.05$) for group D, group C and group B respectively. The results indicate that the hydrolysates with different molecular weights reduced the blood urea nitrogen and enhanced the exercise load. The reduced protein metabolism of the hydrolysate is indicative of enhanced endurance.

Serious exercise will leads to hypoxia, which reduces pyruvate into blood lactic acid, which is an acidic metabolite. The increased blood lactic acid content decreases the pH of tissues, disrupting the acid–base balance and affecting

metabolism, which leads to a decline in athletic ability and muscle contractions (Evans et al. 2002). Therefore, the metabolite of muscle activity, lactic acid is also an important indicator of exercise fatigue (Wang et al. 2006; Ma et al. 2008). The blood lactic acid of mice was determined after swimming. The results were shown in Fig. 5. Compared with the control group, the blood lactic acid in the other three groups decreased, with those in group C and group D decreased significantly ($p<0.05$). Although the blood lactic acid in group B was lower than in group A, the differences were not significant ($p>0.05$).

Sugar is an important source of energy. Sugar is the first source of energy under both short high-intensity exercise and prolonged low-intensity exercise. The body only starts to consume fat and protein once the sugar supply is exhausted. Body sugar includes blood glucose, hepatic glycogen and muscle glycogen (Jung et al. 2007). Hepatic glycogen is only metabolized to maintain blood sugar level after muscle glycogen has been consumed. High hepatic glycogen reserves help prevent the decline in exercise capacity and endurance; hence,

Fig. 5 Effect of hydrolysate on BUN, Blood lactic acid and Hepatic glycogen contents of mice. Data express the mean $\pm$ SD, $n=3$ in each group, * $p<0.05$, ** $P<0.01$, compared with group A (control group)

increasing liver glycogen relieves fatigue (Jia and Wu 2008). As shown in Fig. 5, the hydrolysates with different molecular weights increased the hepatic glycogen content of mice. Group B and group C were significantly different from group A ($p < 0.05$). Group D was highly different from group A ($P < 0.01$). The experimental results indicated that although the molecular weights were different, all of the hydrolysate could contribute to glycogen synthesis and increased the hepatic glycogen content. However, further studies are needed to determine whether highly denatured soybean meal hydrolysate increase the hepatic glycogen content or reduce its consumption during exercise.

The muscle glycogen content and its metabolism determine physical endurance. Many studies found that the amount of muscle glycogen determines physical strength (Williams 2004). Hepatic glycogen is also consumed to maintain balance of blood sugar under increased muscle glycogen consumption. Therefore, the muscle glycogen content also reflects the degree of fatigue (Ren et al. 2011). As shown in Fig. 6, although the muscle glycogen contents of the three treatment groups increased relative to group A (control group), the difference was not significant ($p > 0.05$).

The anti-exercise-fatigue effects of the hydrolysate on mice were evaluated by measuring hemoglobin, blood urea nitrogen, blood lactic acid, hepatic glycogen, and muscle glycogen. Although the increase in muscle glycogen was not significant, all of the other indicators showed that highly denatured soybean meal hydrolysate exhibited an anti-exercise-fatigue effect. Similar to antioxidant activity, the anti-exercise-fatigue effect increased with decreasing molecular weight. Both the antioxidant activity and the anti-exercise-fatigue effect of soy peptides peaked when the molecular weight was less than 5 kDa.

In vivo antioxidant activity

The changes in the SOD activity of the liver homogenate before and after exercise were shown in Table 3. The resting

Table 3 Effect of exercise on hepatic SOD and GSH-PX activity and MDA content of the mice

Group	SOD(U/mgprot)	GSH-PX(U/mgprot)	MDA(nmol·ml ⁻¹)
Group M1	105.28±10.18	120.96±9.95	1.78±0.10
Group M2	91.44±12.07**	100.84±7.38**	1.98±0.16*
Group N1	119.61±9.78**	137.57±12.23**	1.51±0.14**
Group N2	106.92±10.63 [#]	119.6±11.05 ^{##}	1.62±0.23

Data express the mean±SD, $n = 3$ in each group

* $p < 0.05$; ** $P < 0.01$, compared with group M1; [#] $p < 0.05$, ^{##} $P < 0.01$, compared with group N1

SOD activity of group N1 (soybean peptide static group) was significantly higher than that in group M1 (control static group; $P < 0.01$), which indicated that soybean peptide improved SOD activity in mice. The post-exercise SOD activity in group M2 (control exercise group) was highly significantly lower than that in group M1 (control static group; $P < 0.01$). The post-exercise SOD activity in group N2 (soybean peptide exercise group) was significantly lower than that in group N1 (soybean peptide static group; $p < 0.05$). These results indicated that exercise consumed antioxidants and produced excess free radicals, resulting in reduced SOD activity (Sözmen et al. 2001). The differences between the control group and the soybean peptide groups indicated that soybean peptide improved SOD activity in mice and prevented the decrease in SOD activity caused by prolonged exercise.

The GSH-PX activities of the liver homogenates before and after exercise were shown in Table 3. The resting GSH-PX activity in group N1 (soybean peptide static group) was highly significantly higher than that in group M1 (control static group; $P < 0.01$), which indicated that soybean peptides improved GSH-PX activity in mice. The post-exercise GSH-PX activity in group M2 (control exercise group) was highly significantly lower than that in group M1 (control static group; $P < 0.01$). The post-exercise GSH-PX activity in group N2 (soybean peptide exercise group) was highly significantly

Fig. 6 Effect of hydrolysate on muscle glycogen of the mice. Data express the mean±SD, $n = 3$ in each group

lower than that in group N1 (soybean peptide static group; $P < 0.01$). These results indicated that exercise consumed antioxidants and produced excess free radicals, resulting in reduced GSH-PX activity (Tharakan et al. 2005).

The MDA contents of the liver homogenates before and after exercise were shown in Table 3. The resting MDA content in group N1 (soybean peptide static group) was highly significantly lower than that in group M1 (control static group; $P < 0.01$), which indicated that soybean peptides reduced the MDA content of mouse livers. The post-exercise MDA content in group M2 (control exercise group) was significantly higher than that in group M1 (control static group; $p < 0.05$). The post-exercise MDA content in group N2 (soybean peptide exercise group) was higher than that in group N1 (soybean peptide static group), but the difference was not significant ($p > 0.05$). This finding showed that exercise caused the lipid peroxidation of polyunsaturated fatty acids because of the free radicals, which triggered the production of MDA (Inal et al. 2001). The differences between the control group and the soybean peptide groups indicated that soybean peptide inhibited lipid peroxidation.

Determination of antioxidant enzyme activity and MDA contents before and after exercise showed that exercise consumed antioxidants and generated excess free radicals, which reduced antioxidant enzyme activity and increased the MDA content. Intragastrically administering soybean peptide increased the activity of SOD and GSH-PX and lowered the malondialdehyde content after exercise. This result indicated that soybean peptide prevented cell damages from lipid oxidation and mitigated fatigue by scavenging free radicals and inhibiting lipid peroxidation (Ding et al. 2011).

Amino acid composition

The amino acid compositions of the protein hydrolysate might be related to their bioactive activities (You et al. 2011). Glutamic acid or glutamic acid-containing peptides exhibit a strong radical scavenging activity because glutamic acid easily donates protons to the electron in the reaction (Rajapakse et al. 2005). Several amino acids, such as valine and leucine, have generally been considered as antioxidants (Chen et al. 1995; Niranjana et al. 2005). The amino acid composition of highly denatured soybean meal hydrolysate with different molecular weight ranges was shown in Table 4. The above three amino acids composition, group B, C, and D were 30.69 %, 30.60 %, and 32.53 % respectively. These findings might indicate that the antioxidant activity was related to the amino acid compositions and species (Je et al. 2007; Mu et al. 2011). Some amino acid play an role in the regulatory metabolism involved in muscular activity. Guezennec et al. (1998) reported that glutamic acid had a very positive effect on the nervous system and would also be helpful during exercise. Marquezi et al. (2003) reported that aspartic acid was helpful

Table 4 Amino acid composition of soybean peptides with different molecule weight

	Group B composition ^a (%)	Group C composition ^a (%)	Group D composition ^a (%)
Aspartic acid	10.95±0.02 ^d	11.27±0.09 ^c	11.54±0.03 ^b
Threonine	4.36±0.02 ^b	4.21±0.02 ^c	4.25±0.05 ^c
Serine	5.06±0.04 ^c	5.09±0.02 ^c	5.17±0.01 ^b
Glutamic acid	16.31±0.02 ^c	16.07±0.03 ^d	16.84±0.06 ^b
Glycine	3.87±0.06 ^c	4.57±0.04 ^b	3.61±0.08 ^d
Alanine	4.79±0.03 ^b	4.16±0.08 ^c	4.01±0.08 ^d
Cystine	5.06±0.05 ^c	4.92±0.05 ^d	5.28±0.08 ^b
Valine	6.25±0.05 ^d	6.46±0.05 ^c	7.21±0.03 ^b
Methionine	4.41±0.08 ^b	3.92±0.05 ^c	3.25±0.04 ^d
Isoleucine	5.17±0.06 ^d	5.44±0.05 ^c	5.58±0.04 ^b
Leucine	8.13±0.06 ^c	8.07±0.06 ^c	8.48±0.04 ^b
Tyrosine	0.00±0.00 ^c	0.40±0.02 ^b	0.00±0.00 ^c
Phenylalanine	4.51±0.06 ^b	4.37±0.08 ^c	3.82±0.05 ^d
Histidine	3.13±0.06 ^d	3.31±0.09 ^c	3.49±0.05 ^b
Arginine	5.19±0.04 ^d	5.92±0.07 ^c	6.17±0.07 ^b
Proline	5.94±0.03 ^b	5.53±0.04 ^c	5.13±0.04 ^d
Lysine	6.59±0.06 ^b	6.47±0.06 ^c	6.37±0.05 ^c
Content of total amino acid	100	100	100

^a Normalised so that the observed amino acid residues add up to 100 % of the total amino acid residues. The percentage content of amino acid was calculated according to the following formula: The percentage content of amino acid (%) = $\frac{\text{Each amino acid content}}{\text{The total content of amino acids}} \times 100\%$

Means with different letters (b–d) differ significantly ($p < 0.05$)

in the oxidative deamination and could lower the blood ammonia concentration, therefore delaying the occurrence of fatigue. The above two amino acids composition, group B, C, and D were 27.26 %, 27.34 %, and 28.38 % respectively. These findings might suggest that the anti-exercise-fatigue effect was related to the amino acid compositions and species (Wang et al. 2008).

Different amino acids have different bioactive activities. Some amino acids, such as glutamic acid, have antioxidant activity and anti-exercise-fatigue effect (Guezennec et al. 1998; Niranjana et al. 2005). Other amino acids, such as aspartic acid, have anti-exercise-fatigue effect without antioxidant activity (Marquezi et al. 2003). This result indicated that the antioxidant activity and anti-exercise-fatigue effect of highly denatured soybean meal hydrolysate might result from different amino acids.

Conclusions

The results showed that ultrasonic pretreatment effectively improved the antioxidant activity of highly denatured soybean

meal hydrolysate prepared using neutrase. The antioxidant activity and anti-exercise-fatigue effect increased with decreasing molecular weight. Both the antioxidant activity and the anti-exercise-fatigue effect of the hydrolysate peaked when the molecular weight was less than 5 kDa. The results of this study could be used to improve utilization of highly denatured soybean meal and provide a theoretical basis for the development of natural antioxidant and anti-exercise-fatigue substances.

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