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Nutrition

Selenium-biofortified corn peptides: Attenuating concanavalin A—Induced liver injury and structure characterization

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ABSTRACT

The relationship between hepatoprotective effects of selenium-biofortified corn (*Zea mays Linn)* peptides (SeCPs) and its antioxidant ability was evaluated and the structure of SeCPs was identified. SeCPs and corn peptides (CPs) both had good antioxidant ability, and the effect of SeCPs was significantly higher than CPs within a certain concentration range ($P < 0.05$). Additionally, animal experiments indicated that SeCPs (200 mg/kg) had a significantly protective effect against concanavalin A (Con A) induced hepatic lesions, as it significantly declined glutamic-pyruvic transaminase (AST), alanine transaminase (ALT) activities, tumor necrosis factor alpha (TNF-α), interferon (IFN)-γ contents in serum, and malondialdehyde (MDA) contents in liver ($P < 0.05$). Superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities in liver were also significantly increased by SeCPs ($P < 0.05$). The amino acid composition of SeCPs with Mw < 1 kDa was mainly glutamic acid (Glu, 31.18%), leucine (Leu, 21.06%) and alanine (Ala, 13.26%). According to the retention time, the amino acid sequences of 8 selenium-biofortified corn peptides and 29 selenium-free corn peptides were identified. Our results illustrated that the mechanisms of SeCPs against Con A induced hepatic injury in mice may be related to its antioxidant ability and reduction of lipid peroxidation, inhibiting the release of immune factors, such as TNF-α and IFN-γ.

1. Introduction

Hepatitis, induced by virus infection, alcoholism and autoimmune diseases, has become an important public problem in a worldwide scale. It is also a leading cause of chronic liver disease, including cirrhosis and hepatocellular carcinoma [1]. Currently, there are two main types of drugs for viral hepatitis therapy: nucleoside analogues (NUC) and interferon (IFN). NUC could quickly and effectively inhibit virus replication and improve the inflammation of liver tissue. However, long-term treatment of UNC could induce the mutation of virus, which then leads to the drug resistance and secondary hepatic injury [2]. It has also been reported that interferon (IFN)-γ obviously inhibited sperm motility and function [3]. Therefore, it is necessary to find more safe and effective natural products to combat liver damage. Matijin-Su, a dipeptide derivative isolated from a Chinese ethnic drug Matijin, could significantly inhibit hepatitis B virus (HBV) DNA replication and exhibit superior potential of anti-HBV activity than the positive control lamivudine [4,5]. Chromone, derivatived from Halenia elliptica, displayed a strong inhibitory ability against the HBV *in vitro* [6]. Hepatic injury induced by concanavalin A (Con A) is a widely used hepatitis animal model, and its pathogenic mechanism is similar to autoimmune hepatitis (AIH) and viral hepatitis [7,8]. Con A induces hepatocyte apoptosis and necrosis, and results in a decrease of alanine transaminase (ALT) and glutamic-pyruvic transaminase (AST) activity in serum [9]. It has been demonstrated that Con A can promote the secretion of immune factors, such as interferon (IFN)-γ and tumor necrosis factor alpha (TNF-α), which cause the development of hepatitis [10]. Some researches displayed that reactive oxygen species (ROS) was also a crucial factor in Con A induced hepatic injury [11–13].

Selenium-enriched food is regarded as the most effective way for selenium supplementation. Selenium intake afford additional health benefits, such as antioxidant ability [14], hepatoprotective effects [15], protective effects on cardiac tissue [16], and the enhancement of immunity [17]. Selenium-enriched compounds, such as selenium-enriched allophycocyanin [18] and selenium-enriched brown rice proteins [19], presented the characteristics of high antioxidant activity and effective liver protection. Foliar-spraying sodium selenite on plants increased grain selenium content [20], grain yield and antioxidant activity [21]. It has been reported that Se-treated corn (*Zea mays Linn)* exhibited a decrease of nitrate concentration and an increase of pigment contents

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(chlorophylls and carotenoids) [22]. Corn protein is the main by-product in the maize processing industry, which is used for producing coarse feed due to its amino acid composition, color, peculiar taste and inferior water-solubility [23]. Corn peptides (CPs), prepared by the hydrolysate of corn protein, are enriched in alanine (Ala), leucine (Leu), glutamine (Glu) and proline (Pro). Our previous studies indicated that CPs was a good candidate for facilitating alcohol metabolism [24], regulating blood pressure [25] and hepatoprotection effects [26–28].

Since selenium and CPs both possess the features of antioxidant activities and hepatoprotective effects, the primary aim of this study is to evaluate the antioxidant activity of selenium-biofortified corn peptides (SeCPs) and CPs *in vitro*. Secondly, the effects of SeCPs and CPs on attenuating Con A-induced liver injury is to evaluate. The third objective is to find out the existing forms of selenium in SeCPs, amino acid composition and sequence of SeCPs.

2. Materials and methods

2.1. Materials and reagents

Alcalase were purchased from Novozymes Co. (Bagsvaerd, Denmark). AST, ALT, SOD, GPx, MDA, were purchased from Jiancheng Biological Engineering Institute (Nanjing, China). Acetonitrile of HPLC-grade was purchased from Fisher Chemical (Bridgewater, NJ, USA). HPLC-grade Trifluoroacetic acid was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents were analytical grade.

2.2. Cultivation of selenium-enriched corn

Corn seeds were purchased from Henan golden camel agricultural technology Co. Ltd (Zhengzhou, China). Selenium-enriched corn was obtained by foliar-spraying selenium fertilizer (1.5 g Na_2SeO_3 and 42 g sodium alginate dissolved in 15 L water). Spraying was conducted on corn leaf surface every other day from full-bloom stage until a month before the harvest.

2.3. Preparation of SeCPs

Selenium-enriched corn protein was prepared as follows: corn flour, obtained from the maize kernels, was mixed with the alkali alcohol solution (0.1 mol/L NaOH and 95% ethanol, 1:1, v/v) in a ratio of 1:13 (w/v). The mixture was incubated at 45℃ for 2 h, then centrifuged at 4000 rpm for 10 min. The pH value of supernatant was adjusted to 6.3 and then standing for 30 min. Finally, the suspension was centrifuged at 4500 rpm for 10 min, the precipitation was collected and lyophilized as selenium-enriched corn protein.

SeCPs was prepared as follows: 2.86% selenium-enriched corn protein suspensions (w/v) were heated at 90–100℃ for 30 min and cooled to 60℃, and the pH value was adjusted to 8.0. An enzyme/substrate ratio of 0.6% (w/w) of substrate and alcalase was mixed and hydrolyzed for 4 h. After 4 h, the hydrolysates were heated at 100℃ for 10 min to inactivate the enzyme. The mixture was subsequently centrifuged at 3000 g for 10 min. Finally, the supernatant was fractionated through using a molecular weight cut-off 5 kDa (PLCC, Millipore, Billerica, MA, USA), and the < 5 kDa peptides fraction was collected and lyophilized for further study. The preparation method of CPs was the same above.

2.4. Determination of total selenium content and speciation

According to national standard GB 5009.93-2010, total selenium content was measured by hydride generation (HG)-atomic fluorescence spectrometry (AFS-8220, Jitian Instrument Co. Ltd, Beijing, China). The atomic fluorescence spectrometry conditions were set as follows: negative high voltage, 240 V; atomizer height, 8 mm; selenium lamp current, 50 mA; carrier gas flux, 300 mL/min. The forms of selenium were analyzed by atomic fluorescence morphology analyzer (LC-10AT-SA-10, Jitian Instrument Co. Ltd, Beijing, China).

The Se speciation was measured by strong anion-exchange (SAX) HPLC-hydride generation-atomic fluorescence spectrometry (HG-AFS). The model of SAX column was Hamilton PRPX100 column (250 nm × 4.1 mm i.d., 10 μm). The flowing phase was 40 mmol/L $(NH_4)_2HPO_4$ at pH 6.0, with the flow rate 0.5 mL/min. The HG-AFS system was set as the same parameters described above.

2.5. Detemination of antioxidant activity in vitro

2.5.1. Superoxide anion free radical ($O_2^{-\cdot}$) scavenging activity

The $O_2^{-\cdot}$ scavenging activity of SeCPs was determined by modified pyrogallol autoxidation method [29]. The reaction mixture was generated by mixing 4.5 mL of Tris−HCl buffer solution (pH 8.2, 0.1 mol/L), 1 mL EDTA, 1 mL samples and 2.4 mL distilled water and incubated at 25℃ for 10 min. Then, the mixture was shaken up and reacted for 3 min prior to addition of 0.1 mL pyrogallol solution (9 mmol/L, 25℃). At last, 50 μL of ascorbic acid (50 mg/mL) was added to terminate reaction. Tris−HCl buffer solution was used for control. The absorbance was measured at 325 nm (UV-102-02WF, Shimadzu, Japan). The $O_2^{-\cdot}$ radical scavenging activity of the sample was evaluated with the following equation:

$$O_2^{-\cdot} \text{ scavenging activity (\%)} = (A_{control} - A_{sample}) / A_{control} \times 100\%$$

2.5.2. Hydroxyl radical ($^{\cdot}OH$) scavenging activity

The $^{\cdot}OH$ scavenging activity of SeCPs was measured by deoxyribose-iron system method [30]. The reaction solution was generated by mixing 0.4 mL phosphate buffer (pH 7.5, 50 mmol/L), 0.1 mL sample, 0.1 mL ethylene diamine tetraacetic acid (1.04 mmol/L), 0.1 mL H_2O_2 (10 mmol/L), 0.1 mL of deoxyribose solution (60 mmol/L), 0.1 mL ascorbic acid (2 mmol/L) and 0.1 mL $FeCl_3$ (1 mmol/L). After incubating the reaction solution at 37℃ for 1 h, 1 mL of hydrochloric acid (25%, v/v) was added to terminate reaction. Then, the solution was mixed with 1 mL 2-thiobarbituric acid (1%) followed by incubating at 100℃ for 15 min. The absorbance of sample was read immediately at 532 nm when sample has cooled down. Distilled water instead of the sample was used for control. The scavenging activity of SeCPs was evaluated by the following equation:

$$^{\cdot}OH \text{ scavenging activity (\%)} = (A_{control} - A_{sample}) / A_{control} \times 100\%$$

2.5.3. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The measurement method of DPPH radical scavenging activity was as follows [31]: DPPH was dissolved in ethanol to a final concentration of 0.1 mmol/L and stored at 4℃ for further study. 1.5 mL distilled water, 1.5 mL ethanol and 1.5 mL sample were mixed with 1.5 mL DPPH (0.1 mmol/L), respectively. The absorbance values (A_0, A_1, A_2, respectively) was recorded at 517 nm. The scavenging rate of DPPH radical of SeCPs was calculated by the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [1 - (A_1 - A_2)/A_0] \times 100\%$$

2.5.4. Reducing power

The reducing power of SeCPs was carried out according to the method of [32]. 2 mL samples were mixed with 2 mL phosphate buffer (0.2 mol/L, pH 6.6) and 2 mL of potassium ferricyanide (1%). The mixture was incubated at 50℃ for 20 min, and then 2 mL of trichloroacetic acid (10%) was added. The mixture was centrifuged at 3000 r/min for 10 min. 2 mL supernate was collected into a tube and

mixed with 2 mL distilled water and 0.4 mL of $FeCl_3$ (0.1%). After 10 min, the absorbance was read at 700 nm.

2.5.5. Inhibition of linoleic acid peroxidation

The capacity of inhibiting linoleic acid peroxidation of SeCPs was measured by ammonium thiocyanate colorimetry method [33]. The linoleic acid emulsion was generated by mingling 0.2840 g linoleic acid, 0.2804 g tween-20 with 10 mL phosphate buffered solution (pH 7.0) and then diluting with distilled water to 50 mL. Sample was dissolved in 0.5 mL distilled water and mixed with 2.5 mL of linoleic acid emulsion. The mixture was incubated at 37℃ for 45 h. Then, 0.1 mL mixed solution was mingled successively with 4.7 mL alcohol (75%), 0.1 mL ammonium thiocyanate (30%) and 0.1 mL of ferrous chloride (0.02 mol/L). After 3 min, the absorbance was measured at 510 nm. Linoleic acid instead of the sample was used for control. The capacity of inhibiting linoleic acid peroxidation was evaluated with the following equation:

$$\text{Capacity of inhibiting linoleic acid peroxidation (\%)} = [1 - (A_{sample}/A_{control})] \times 100\%$$

2.6. Animals and experimental design

Seventy male BALB/c mice (19 ± 1 g), SPF, were obtained from Hubei Laboratory Animal Center (Hubei, China). All procedures were conducted according to the National Institute of Health Guidelines for the Care and Use of Animals and the animal ethical approval certificate number was MU-2016-001. After 3 days of acclimation, mice were randomly divided into seven groups: Normal, Model, Na_2SeO_3, Na_2SeO_3 + CPs, CPs, SeCPs and Silymarin. Each group was maintained at standard environmental conditions with 12 h dark-light cycles, and had free access to commercial food (Composition (g/kg): corn flour 500; wheat bran 90; wheat flour 90; bean dregs 220; fish flour 70; sodium chloride 5; multivitamins 1; trace elements 1.6; bone meal 20) and water. The test dosages of Con A, CPs and SeCPs were decided by preliminary tests. Normal and model group were intragastric administrated with normal saline (10 ml/Kg bw) for 14 days. Na_2SeO_3, Na_2SeO_3 + CPs, and SeCPs group all received 0.58 μg/Kg bw selenium (0.58 μg/Kg bw Na_2SeO_3, 0.58 μg/Kg bw Na_2SeO_3 + 200 mg/Kg bw CPs, 200 mg/Kg bw SeCPs (selenium content was 0.58 μg/Kg), respectively) by administered intragastrically for 14 days. CPs and silymarin group received 200 mg/Kg bw CPs and 50 mg/Kg bw silymarin by gavage for 14 days. Four hours after the last intragastric administration, mice were injected 15 mg/Kg body weight Con A into the veins of the tails. Normal group was given the same volume normal saline by tail vein injection. After an 12 h fasting period, all mice were sacrificed by cervical dislocation. Blood, liver, spleen and thymus were removed for further tests.

2.6.1. Serum parameters

Serum ALT and AST activities were measured by detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The concentration of serum INF-γ and TNF-α were determined by using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols.

2.6.2. Liver parameters

Liver homogenate was prepared by mixing liver sample and stroke-physiological saline solution at the ratio of 1:9 (w/v). The liver MDA contents, SOD and GPx activity were analyzed by detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.7. Analysis of amino acid composition

The lyophilized sample was digested at 110℃ for 24 h with HCl (6 mol/L). The amino acid analyzer (835-50, Hitachi, Tokyo, Japan) was used to determine the amino acid composition of the SeCPs.

2.8. HPLC analysis

SeCPs with a molecular weight of less than 1 kDa were performed on Waters e2998 HPLC-DAD system (Waters, Milford, MA, USA). 10 mg/mL sample was analyzed on a Zorbax SB-C18 column (250 mm × 4.6 mm, 0.5 μm, Agilent Co., Santa Clara, CA, USA) with a flow rate maintaining at 1 mL/min. A binary gradient elution system was composed of ultrapure water (with 0.1% TFA, A) and acetonitrile (with 0.1% TFA, B). Gradient elution conditions were as follows: 0 − 60 min, A (95 − 65%), B (5 − 35%). The volume of injection was 30 μL, and the DAD detector was set at 220 nm for analysis.

2.9. HPLC-ESI-MS/MS analysis

Chromatographic conditions were the same as above in the HPLC analysis. Mass spectrometry conditions were set as follows: ESI^+ ion source; spray voltage, 3500 V; spray gas (N_2) pressure, 275.79 kPa (40 psi), dry gas (N_2) flow rate, 10.0 L/min; capillary temperature, 365℃; mass-to-charge ratios, 50 − 1000. LCMSD-Trap Data Analysis software was used to identify the sequences. The sequences were confirmed by searching on the UCSF Mass Spectrometry Facility (http://prospector.ucsf.edu/prospector/cgibin/msform.cgi?form=mspattern).

2.10. Statistical analysis

All results were presented as mean values ± (SD). The statistical analysis was performed with SAS software ver.8.1 and carried out one-way analysis of ANOVA with Duncan's multiple range test and $P < 0.05$ or $P < 0.01$ values were considered as statistically significant.

3. Results and discussions

3.1. The total Se content and Se speciation in SeCPs

The total selenium content increased from 2.19 mg/kg (corn kernel) to 12.97 mg/kg (corn protein). The total selenium content was further improved to 32.37 mg/kg after the hydrolysis by alcalase (data was not shown here). As shown in Table 1, 37.47% and 30.9% of selenium existed in the molecular weight of 1–3 kDa, 3–5 kDa, respectively, which demonstrated that selenium was evenly distributed in each fraction.

Different samples pretreatment methods have marked influence on the forms of selenium. Common samples preparation methods are extraction, volatile enrichment, and enzymolysis method [34]. The selenocysteine (SeCys) and selenomethionine (SeMet) were detected in SeCPs, while inorganic selenium was not detected (data was not shown). This result demonstrated that the selenium of SeCPs was mainly in forms of organic selenium. R. Lobinski (2007) used a successful multidimensional method for both the qualitative and quantitative characterisation of a SeCys-rich aqueous fraction of selenised

Table 1
Selenium content in different SeCPs fractionation (n = 5).

Fraction	< 1 kDa	1-3 kDa	3-5 kDa
Separated peptide quality(g)	0.76	1.87	1.87
Selenium quality(μg)	19.85	60.64	50.02
The proportion of selenium.(%)	12.26	37.47	30.90

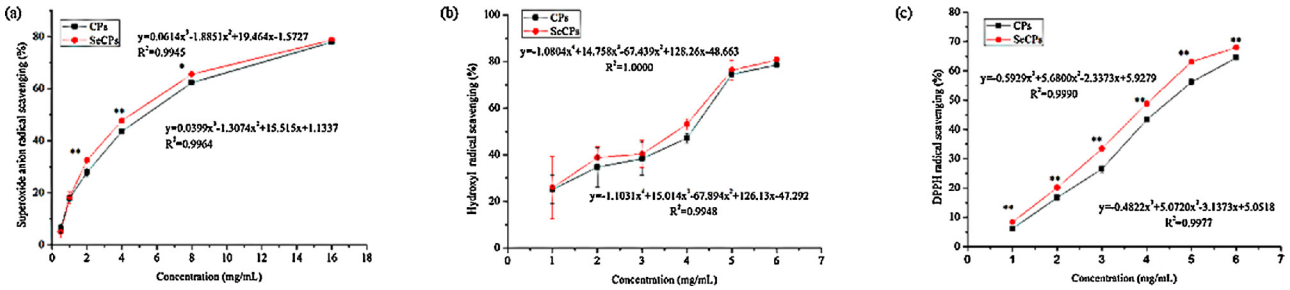

Fig. 1. Scavenging effect of different concentrations of SeCPs and CPs (n = 3). (a) superoxide anion free radical scavenging activity; (b) hydroxyl radical scavenging activity; (c) DPPH radical scavenging activity. * within column means significantly different, $P < 0.05$ (Duncan's multiple range test at $P < 0.05$). ** within column means highly significantly different, $P < 0.01$ (Duncan's multiple range test at $P < 0.01$).

yeast. They firstly used Size-exclusion chromatography (SEC)–strong anion-exchange (SAX) HPLC-ICP-MS to separate different Se species and then quantitated. Then the structures were identified by reversed phase (RP) nano-HPLC-electrospray Q-TOFMS/MS [35]. Chaudhary (2010) reported that organic selenium possessed 120–200% bioavailability compared to sodium selenium in guinea pig [36].

3.2. In vitro antioxidant activity of SeCPs

3.2.1. Superoxide anion radical scavenging activity

$O_2^{-\cdot}$ is the root cause of free radicals, which can generate ·OH and H_2O_2 in disproportionated reaction. $O_2^{-\cdot}$ scavenging activity can be used as an important indicator of its antioxidant activity [37]. As shown in Fig. 1a, SeCPs had significantly higher $O_2^{-\cdot}$ scavenging activity than CPs at the concentration of 2–8 mg/mL ($P < 0.05$). This results showed that selenium enhanced the $O_2^{-\cdot}$ scavenging ability of peptides, which was in accordance with Liu et al [38] that the Se-enriched brown rice protein displayed superior antioxidant activity than Se-free samples. After the polynomial regression analysis, the IC_{50} value of CPs (4.82 mg/mL) on superoxide anion free radical was significantly higher than that of SeCPs (3.99 mg/mL) ($P < 0.05$). These results demonstrated that SeCPs had significant effects on $O_2^{-\cdot}$ scavenging ability and might be used as potential antioxidants.

3.2.2. Hydroxyl radical-scavenging activity

·OH is the most active chemical property of reactive oxygen. Excessive free radical can react with a majority of biomacro-molecules and then trigger a series of free radical chain reactions. As demonstrated in Fig. 1b, SeCPs and CPs both had ·OH scavenging activity. ·OH scavenging ability of the corn peptides was improved after selenium-biofortified, whereas there was no significant difference observed between SeCPs and CPs ($P > 0.05$). Polynomial regression analysis indicated that the IC_{50} values of SeCPs and CPs on hydroxyl radical were 3.83 mg/mL, 4.03 mg/mL, respectively, which was no significant difference between two groups ($P > 0.05$).

3.2.3. DPPH radical scavenging activity

The DPPH radical scavenging activity of SeCPs was significantly higher than CPs at the same dose ($P < 0.01$, Fig. 1c), which showed that selenium in SeCPs played an important role in DPPH radical scavenging activity. The activity of SeCPs was further enhanced to 50% at the concentration of 4 mg/mL. In comparison, SeCPs showed a superior scavenging effects than the selenium-containing polysaccharides (46.45% at 5 mg/mL) reported by Wang et al [39].

3.2.4. Reducing power

Reducing power is an indicator of the electron-donating capacity of antioxidant, which can indirectly determine the antioxidant capacity of SeCPs. As demonstrated in Fig. 2a, there was no difference between SeCPs and CPs under the concentration of 4 mg/mL ($P > 0.05$). At the dose of 5 mg/mL and 6 mg/mL, the reducing power of SeCPs was significantly higher than CPs ($P < 0.01$). This results showed that the reducing power contributed nothing to the antioxidant capacity of SeCPs at low concentration. Sulfur of sulfhydryl was substituted by selenium in Cys/Met after the Se-treatment. As for the electron acquisition capability of selenium was stronger than sulfur, the ability of SeCys/SeMet to provide electron was weaker than Cys/Met. Thus, Se-biofortified did not significantly enhance the reducing power ability than Se-free peptides in low concentration.

3.2.5. Inhibition of linoleic acid peroxidation

The reaction between free radical and unsaturated fatty acid can be weaken by antioxidants, and then the processes of lipid peroxidation are also reduced [40]. Fig. 2b showed that SeCPs and CPs exhibited the lipid peroxidation activity in a concentration dependent relationship. The peroxidation inhibition activity of SeCPs was significantly higher than CPs at the same concentration ($P < 0.05$). Polynomial regression analysis indicated that the IC_{50} value of CPs (7.57 mg/mL) in linoleic acid was significantly higher than that of SeCPs (5.58 mg/mL) ($P < 0.05$).

Selenium (Se) is an essential micronutrient that enhances antioxidant activity and anti-inflammatory in human and animals via selenoproteins [41]. Glutathione peroxidase1 (GPx1) is first identified and the most abundant selenoprotein in mammals [42,43]. GPx is expressed in both cytosol and mitochondria and can reduce H_2O_2 and organic hydroperoxides levels to protect cells from oxidative injury [42]. Epidemiological evidence indicates that high Se intake effectively reduce the risk of mammary, prostate, lung, colon, neuro-oncology and liver cancer [44].

3.3. Hepatoprotective effects of SeCPs

3.3.1. Effects of SeCPs on organ indexes

Organ indexes reflect nutritional state and visceral lesion of laboratory animals. As shown in Table 2, the liver index and spleen index were significantly higher ($P < 0.05$), while there was no significant difference on thymus index in model group compared to the normal group ($P > 0.05$). After the supplement of Na_2SeO_3 + CPs, CPs, SeCPs and silymarin, the liver index was decreased significantly ($P < 0.05$), which illustrated that Na_2SeO_3 + CPs, CPs, SeCPs and silymarin attenuated Con A induced liver injury. Silymarin, a natural compound derived from the species *Silybum marianum*, is almost universally recognized as a liver protection drug due to its inhibition of the free radicals [45]. Moreover, thymus is one of the important immune organs of the animal, and thymus index is an indicator to relatively evaluate the whole immune state of the organism. The thymus index in SeCPs group was significantly higher compared to the model group ($P < 0.05$), which was in accordance with the effect of Zhuyeqing Liquor [46]. Additionally, the effect of SeCPs was better than silymarin. These data demonstrated that SeCPs may play a role in attenuating the organ damage in the immunological liver lesion model.

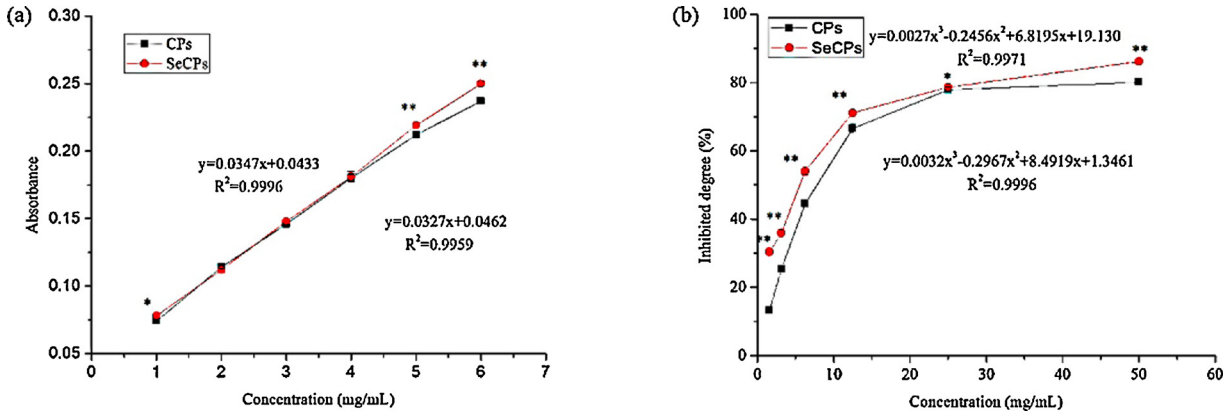

Fig. 2. Reducing power and Inhibition rates of different concentrations of SeCPs and CPs (n = 3): (a) reducing power; (b) inhibition rates in linoleic acid auto-oxidation system. * within column means significantly different, $P < 0.05$ (Duncan's multiple range test at $P < 0.05$). ** within column means highly significantly different, $P < 0.01$ (Duncan's multiple range test at $P < 0.01$).

Table 2
Effects of SeCPs on organ indexes of Con A-induced mice (n = 10).

Group	Liver Index (mg/g)	Spleen Index (mg/g)	Thymus Index (mg/g)
Control	41.47 ± 3.23^{d}	4.40 ± 0.74^{c}	2.38 ± 0.28^{a}
Model	54.29 ± 1.43^{a}	7.72 ± 0.68^{a}	2.03 ± 0.13^{a}
Na_2SeO_3	51.20 ± 1.43^{b}	7.64 ± 0.48^{a}	2.09 ± 0.37^{a}
Na_2SeO_3 + CPs	49.73 ± 1.83^{bc}	7.09 ± 0.87^{a}	2.58 ± 0.65^{a}
CPs	50.20 ± 1.44^{bc}	7.30 ± 0.67^{a}	2.37 ± 0.67^{a}
SeCPs	49.49 ± 3.21^{bc}	6.86 ± 0.97^{a}	2.79 ± 0.83^{b}
Silymarin	47.75 ± 1.95^{c}	5.86 ± 0.74^{b}	2.53 ± 0.80^{a}

3.3.2. The values of AST, ALT, INF-γ, TNF-α in serum

ALT and AST are vital indicators of liver diseases, and they massively exist in the liver. As shown in Fig. 3ab, significant increases ($P < 0.05$) of AST and ALT activity were observed in model group compared to the normal group. Additionally, AST and ALT activity were markedly decreased in Na_2SeO_3 + CPs, CPs, SeCPs and silymarin groups compared to the model group ($P < 0.05$). These results indicated that the effects of SeCPs, CPs and Na_2SeO_3 + CPs pretreatment were superior than Na_2SeO_3 pretreatment in providing protection against Con A induced oxidative damage.

The activation of T-cells and subsequent release of inflammatory factors, such as INF-γ and TNF-α, were promoted after Con A induced liver injury [47]. The concentration of serum INF-γ in the model group was significantly higher compared to the control group ($P < 0.05$, Fig. 3c). After the supplement of Na_2SeO_3 + CPs, CPs, SeCPs and silymarin, the concentration of serum INF-γ was significantly decreased ($P < 0.05$). As shown in Fig. 3d, the concentration of serum TNF-α in Na_2SeO_3 + CPs, SeCPs and silymarin groups recovered to the normal levels and there was no significant difference observed among these three groups ($P > 0.05$). These data indicated that SeCPs could effectively antagonize the Con A induced hepatic damage by modulating of the levels of INF-γ and TNF-α. It has been reported that CPs was an immune regulator, that could effectively inhibit the hepatocellular carcinoma in H22-bearing mice via the enhancement of the body immune system function [48]. Pretreatment of ginsenoside Rg1 also markedly declined the level of inflammatory factors, such as IL-6, INF-γ and TNF-α, and then relieved the liver lesion [49].

3.3.3. Effects of SeCPs on hepatic SOD, MDA and GPx activity

Oxidative stress is related to various liver damages, such as fatty liver disease, drug-induced liver disease and virus hepatitis, which damage the hepatocellular antioxidant defense system functions by reducing enzymatic components such as SOD, GPx. As shown in Fig. 4abc, MDA content was significantly decreased in Na_2SeO_3, Na_2SeO_3 + CPs, CPs, SeCPs and silymarin groups compared to the model group ($P < 0.05$). Significant enhancement of SOD activity was observed in SeCPs and silymarin groups compared to the model group ($P < 0.05$), while there was no significant difference between these two groups ($P > 0.05$). The GPx activity was significantly higher in SeCPs group compared to Na_2SeO_3 group ($P < 0.05$), which indicated that the effect of SeCPs was superior than Na_2SeO_3.

Shin et al. reported that a traditional Korean medicine CGX inhibited the liver damages induced by Con A *via* the significant reduction of lipid peroxidation and GSH content [11]. It was also reported that andrographolide attenuated the expressions of oxidative stress response genes, and then reduced oxidative stress response in the liver [13]. In this study, SeCPs significantly elevated liver SOD and GPx activity, which led to the decrease of oxidative stress response and the inhibition of hepatic injury.

3.4. The amino acid composition of SeCPs

The amino acid composition of SeCPs with Mw < 1 kDa was determined and presented in Table 3. Results showed that Ala and Leu were abundant in SeCPs, accounting for 13.26% and 21.06%, respectively. Leu and Ala are hydrophobic amino acid, which have effects on facilitating alcohol metabolism [7], hepatoprotective effect and antioxidant activity [50,51]. Leu could stimulate the translation of mRNA to regulate the protein metabolisms [52]. Hydrophobic peptides with high lipophilicity can easily transfer through lipid barrier of intestinal mucosal cell. Hydrophobic amino acid contents in SeCPs accounted for about 50% of total amino acid, and further certified that SeCPs had favourable hepatoprotective effect. In addition, the branched chain amino acids (BCAAs) contents in SeCPs constituted 24.99%. The catabolism of BCAAs was a regulator of physiological aging process. BCAAs participated in many physiological and pathological processes, especially the preventive and therapeutic effect of hepatic diseases [53,54]. When happened to liver lesions, BCAAs in serum accelerated oxidation and then in a state of deficiency. If BCAAs were supplemented in time, hepatic damage was then relieved. In high-fat diet (45%), BCAAs reduced triglyceride in liver [55]. In a choline-deficient high-fat diet, which induced nonalcoholic fatty liver disease, BCAAs improved fatty liver and slowed down the processes of disease [56]. It has been reported that the supplementation of BCAAs, especially Leu, not only inhibit the CCl_4-induced liver fibrosis [57], but also improve the quality of life in patients with liver cirrhosis [58].

3.5. Structure summary of SeCPs (< 1 kDa)

Since peptide sequence is of great significance in deciding the

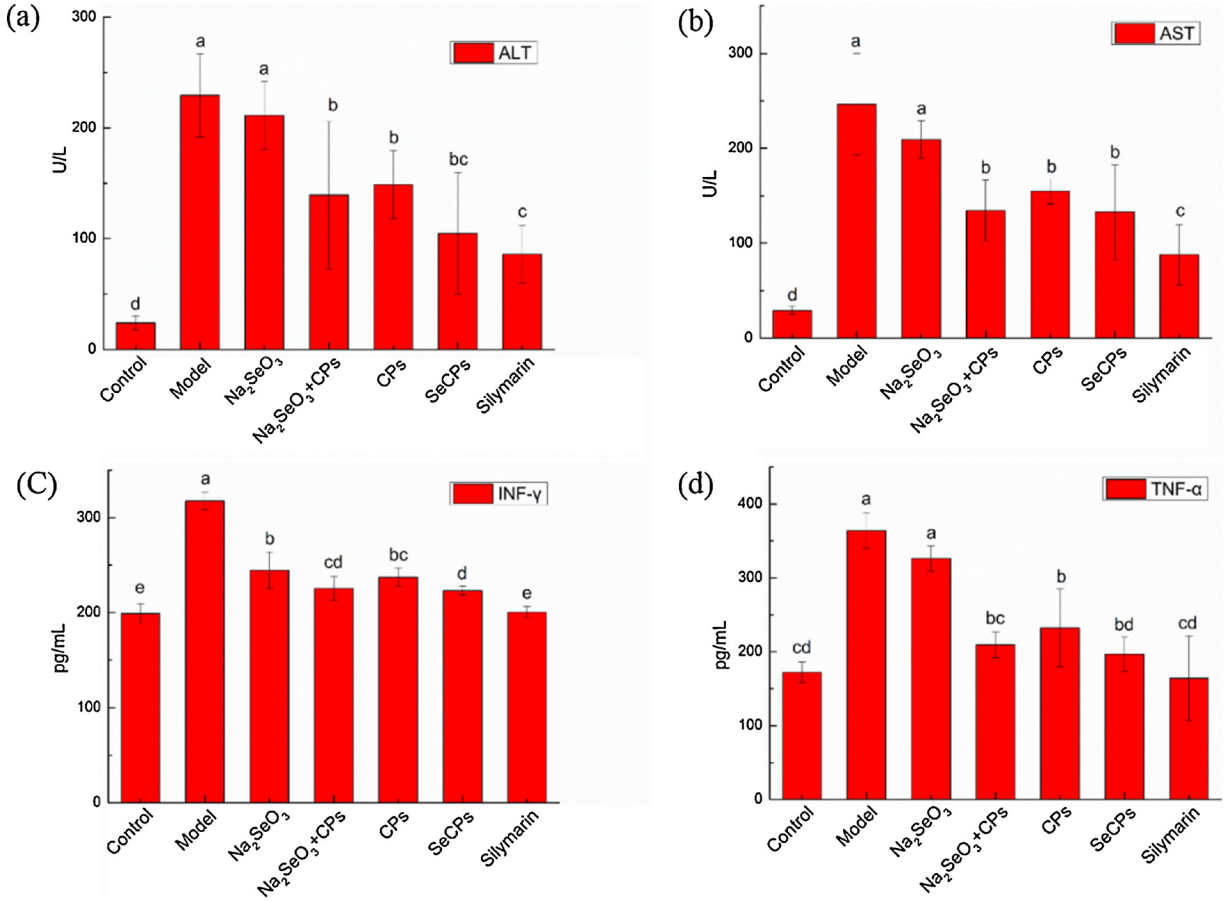

Fig. 3. Effects of each group on the activities of serum (a) alanine aminotransferase (ALT), (b) aspartate aminotransferase (AST), (c) interferon-γ (INF-γ) and (d) tumor necrosis factor alpha (TNF-α). Mean values with different letters are significantly different at P<0.05.

antioxidant ability and hepatoprotective effects of bioactive peptides, it is inevitable to know the exact structures of SeCPs. According to the retention time, the amino acid sequences of 8 selenium-biofortified corn peptides (Table 4) and 29 selenium-free corn peptides (data was not shown here) were identified. The selenium-enriched peptides were Phe-SeCys, Trp-SeMet-Gln-Glu, Trp-SeMet-Lys-Glu, SeCys-Tyr-Glu, Phe-MeSeCys-Met, SeCys-Cys-Ala-MeSeCys, Phe-MeSeCys-Val-Gln and Phe-MeSeCys-Val-Lys, respectively. The results indicated that selenium was in forms of SeCys, SeMet and methyl selenocysteine (MeSeCys) in selenium-biofortified corn peptides, and selenium-biofortified corn peptides presented the characteristics of high contents of hydrophobic amino acid, such as tryptophan (Trp), methionine (Met), valine (Val), Ala and phenylalanine (Phe), which accounted for 50% of total amino acid. It has been reported that the high antioxidant activities were related to high hydrophobic amino acids or peptides [59]. Liu et al. identified the sequence SeMet-Pro-Ser with strong antioxidative activities [38]. Most of selenium-free corn peptides were tetrapeptide and pentapeptide, which were also rich in hydrophobic amino acid and BCAAs.

4. Conclusions

SeCPs have good antioxidant abilities against DPPH$^{\cdot}$, OH^{-} and $O_2^{-\cdot}$. The pretreatment of SeCPs could effectively antagonize the Con A induced liver injury via its antioxidant ability, and selenium and CPs have synergistic effects to some extent. Eight selenium-enriched peptides are identified by HPLC-ESI-MS/MS, and SeCys, SeMet and MeSeCys are the main forms of organic selenium in selenium-biofortified corn peptides. The exact mechanism of the liver protective effect of SeCPs and structure-activity relationship will be further studied.

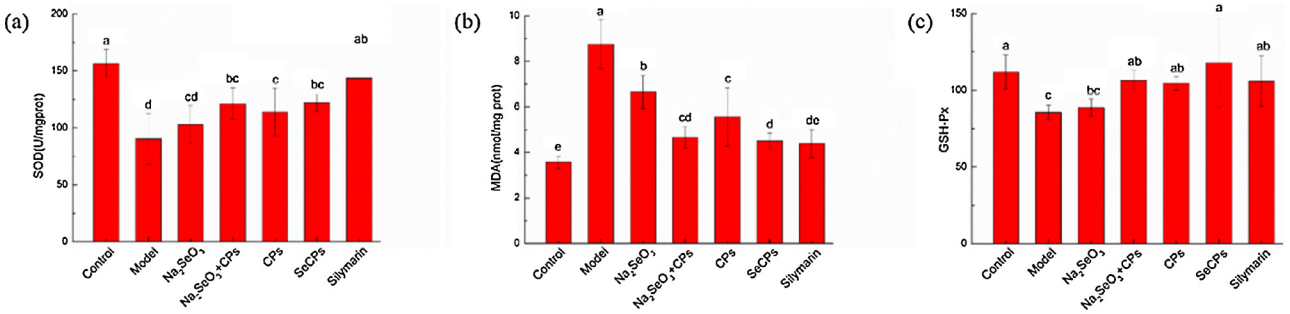

Fig. 4. Effects of each group on the activities of hepatic (a) superoxide dismutase (SOD), (b) malondialdehyde (MDA) and (c) glutathione peroxidase (GPx). Mean values with different letters are significantly different at P<0.05.

Table 3
Amino acid composition of SeCPs (MW < 1 kDa).

Amino acid	SeCPs (mg/100 mg)	Amino acid	SeCPs (mg/100 mg)
[a]Asx (D + N)	2.78	Leu (L)	13.3
Thr (T)	1.33	Tyr (Y)	1.89
Ser (S)	2.29	Phe (F)	2.66
[b]Glx (E + Q)	19.69	Lys (K)	0.38
Gly (G)	1.17	His (H)	0.6
Ala (A)	8.37	Arg (R)	0.94
Pro (P)	0.6	Hyp	3.51
Val (V)	1.29	Total branched chain amino acid am	15.78
Met (M)	0.95	Total hydrophobic amino acid a	31.27
Ile (I)	1.19	Total amino acid	63.14

[a] Asx: Asp + Asn (D + N).
[b] Glx: Glu + Gln (E + Q).

Table 4
Structure summary of Se-containing corn peptides analysis.

Retention time (min)	Sequence	Protein name	Sequences of peptide (the proportion of hydrophobic amino acid)
5.9	FC	MAIZE	F-SeC(50%)
8.1	WMQE	MAIZE	W-SeM-Q-E(50%)
8.1	WMKE	ZEALU	W-SeM-K-E(50%)
8.1	WMKE	MAIZE	W-SeM-K-E(50%)
8.1	WMKE	ZEADI	W-SeM-K-E(50%)
8.1	WMKE	ZEAMP	W-SeM-K-E(50%)
10.4	CYE	MAIZE	SeC-Y-E(0%)
10.4	CYE	ZEAMP	SeC-Y-E(0%)
10.4	FSM	MAIZE	F-MeSeC-M(67%)
11.5	CCAC	MAIZE	SeC-C-A-MeSeC(25%)
11.5	CCAC	ZEAMP	SeC-C-A-MeSeC(25%)
50.5	FCVQ	MAIZE	F-MeSeC-V-Q(50%)
50.5	FCVQ	ZEAMP	F-MeSeC-V-Q(50%)
50.5	FCVK	ZEAMP	F-MeSeC-V-K(50%)
50.5	FCVK	MAIZE	F-MeSeC-V-K(50%)
50.5	FCVK	ZEAMM	F-MeSeC-V-K(50%)

The primary structures of the SeCPs (< 1 kDa) were determined and validated by searching three mass spectrometry databases of SwissProt.2013.6.27, NCBInr.2013.6.17, UniProtKB.2013.6.17 with the Protein Prospector engine.

Conflict of interest

The authors declare that there are no conflicts of interest.

Author contribution

Danjun Guo, Tao Hou and Hui He proposed and designed the experiment. Danjun Guo, Yan Zhang, Juanjuan Zhao, Hui He all participated in the experiment. Danjun Guo analyzed the data and wrote the manuscript. Tao Hou revised the manuscript and was responsible for the supervision of the whole research.

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