Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.61522

Hepatoprotective effect of clam (*Corbicula fluminea*) protein hydrolysate on alcohol-induced liver injury in mice and partial identification of a hepatoprotective peptide from the hydrolysate

Jialong GAO[1,2,3,4,5,6], Chaohua ZHANG[1,2,3,4,5,6*], Xiaoming QIN[1,2,3,4,5,6], Wenhong CAO[1,2,3,4,5,6], Jianping CHEN[1,2,3,4,5,6], Yujin LI[7*], Huina ZHENG[1,2,3,4,5,6], Haisheng LIN[1,2,3,4,5,6], Zhongqin CHEN[1,2,3,4,5,6]

Abstract

In the current research, edible meat of freshwater clam *Corbicula fluminea* was subjected to enzymatic peptide extraction using animal protease. The clam protein hydrolysate (CPH) contained 45.78% essential amino acids and 44.97% hydrophobic amino acids and showed high alcohol dehydrogenase (ADH) activation in vitro. Four fractions were divided from CPH by ultrafiltration and further investigated for their hepatoprotective effects on alcohol-induced injury in mice. The 2.5-5 kDa fraction (CPH-3) showed the best effect for preventing hepatic damage caused by alcohol, as revealed by reduced serum aspartate transaminase (AST) and alanine transaminase (ALT) activities and hepatic malondialdehyde (MDA) level, the marked decrease in the hepatic triglyceride (TG) contents, and the enhanced hepatic ADH, aldehyde dehydrogenase (ALDH), and glutathione (GSH) activities. Furthermore, histopathology of CPH-3-treated mice showed less hepatic tissue damage compared to alcohol- or other CPHs-treated mice. Hence, CPH-3 was further purified by consecutive chromatographic techniques with gel filtration and reverse-phase high-performance liquid chromatography. One hepatoprotective peptide, KKLAGFLRYYS (1330.68 Da), was identified by LC-MS/MS, which exhibited strong ADH activation activity. Results suggested that CPH could be a potential nutraceutical for facilitating alcohol metabolism and preventing or ameliorating early liver injury induced by acute alcohol exposure and that the peptide KKLAGFLRYYS is one of the main bioactive components in CPH.

Keywords: *Corbicula fluminea*; protein hydrolysates; hepatoprotective effect; alcohol-induced liver injury; peptide.

Practical Application: Freshwater clam *Corbicula fluminea* is widely distributed in East Asian countries, especially in China. The study shows clam protein hydrolysate and its isolated peptide KKLAGFLRYYS have a good hepatoprotective effect.

1 Introduction

Heavy alcohol consumption has become a serious public health problem worldwide, which contributed to approximately 3 million deaths (5.3% of all deaths) and more than 5% of global disease burden in 2016 (World Health Organization, 2018). China has a long history of drinking alcoholic beverages, and the alcohol consumption has become more and more severe in recent years, thus increasing the incidence of alcoholic liver disease (ALD), which is considered the primary cause of morbidity and mortality worldwide (Feng et al., 2019). A growing body of evidence has indicated that ALD in the early stage is reversible (Bai et al., 2020; Wang et al., 2014). Consequently, the development of new strategies for preventing or slowing down the development of ALD in the early stage is meaningful. Over the past decades, owing to their multiple targets and fewer side-effects than most pharmaceuticals, effective natural compounds for the prevention and/or treatment of ALD have become a popular research focus.

Bioactive peptides are derived from food proteins and exert a positive effect on humans due to their health-promoting properties (Shori et al., 2021). Bioactive peptides can exert several beneficial effects like preventing diseases or modulating physiological systems once they are absorbed into the human body (Toldrá et al., 2018). Since studies first reported that corn peptides could lower blood alcohol level by elevating plasma alanine and leucine (Yamaguchi, 1998), the production and properties of bioactive peptides with functions related to reducing ALD have been reviewed in many recent articles. Accordingly, peptides originating from corn (Ma et al., 2012), chum salmon skin collagen (Lin et al., 2012), blue mussel (Park et al., 2016), krill (Park et al., 2019), snapper fish scale (Chen et al., 2020), chicken liver (Lin et al., 2017), and chicken breast muscle (Xiao et al., 2018) have been found to be hepatoprotective.

Received 07 June, 2022
Accepted 22 July, 2022
[1] *College of Food Science and Technology, Guangdong Ocean University, Zhanjiang, China*
[2] *National Research and Development Branch Center for Shellfish Processing, Zhanjiang, China*
[3] *Guangdong Provincial Key Laboratory of Aquatic Products Processing and Safety, Zhanjiang, China*
[4] *Guangdong Province Engineering Laboratory for Marine Biological Products, Zhanjiang, China*
[5] *Key Laboratory of Advanced Processing of Aquatic Product of Guangdong Higher Education Institution, Zhanjiang, China*
[6] *Collaborative Innovation Center of Seafood Deep Processing, Dalian Polytechnic University, Dalian, China*
[7] *College of Food Science and Engineering, Ocean University of China, Qingdao, China*
*Corresponding author: Zhangch2@139.com; liyujin@ouc.edu.cn

Corbicula fluminea is a freshwater bivalve mollusk widely distributed in East Asian countries, especially in China. It has high edible value and has long been used as a folk remedy in Chinese tradition, due to health functions such as being appetizing, purging milk, brightening eyes, diuresis, removing toxins, reducing fever, relieving cough and phlegm, treating measles, and as anti-alcoholism medicine (Wang et al., 2019). The first description of the hepatoprotective activity of freshwater clam was in "Bencao Gangmu", written by Li Shizhen in 1578. To date, modern pharmacological studies have shown the hot-water extract of freshwater clam (FCW) significantly decreased the intensity of chemical-induced hepatic damage in a rodent model (Chijimatsu et al., 2015; Hsu et al., 2010), FCW or the hydrolysate of its residual clam meat alleviated non-alcoholic fatty liver disease in tilapia fed with high-fat diet (Lin et al., 2018), and the extract of freshwater clam (including FCW and clam residual meat) reduced steatohepatitis and liver injury of mice induced by high-fat, high-cholesterol, and cholic acid diet (Yao et al., 2018). The multiple hepatoprotective effects of *C. fluminea* can be due to its chemical ingredients, such as proteins, amino acids, sugar, fatty acids, and trace elements, and especially the protein, which is supposed to be the most important nutrient of the human body, and accounted for 53.4-62.8% of FCW (Chijimatsu et al., 2015; Huang et al., 2013). Considering protein absorption in the human body is mainly in the form of free amino acids or oligopeptides, we thus hypothesized that clam protein peptide may be beneficial for the prevention of alcohol-induced liver injury. Therefore, the main objective of this study was to evaluate the hepatoprotective effect of clam protein hydrolysate (CPH) and then to purify clam hepatoprotective peptide from the CPH.

2 Materials and methods

2.1 Preparation of clam protein hydrolysate (CPH)

Freshwater clams *C. fluminea* were purchased from the local Huangsha Market, Guangzhou, China. They were transported to the laboratory on ice in insulated boxes. The edible meat (approximately 1000 g) was separated from the shells and stored at −20 °C until use, and its crude protein content was 58.1% (dry basis) by the Kjeldahl method. Clam meat was mixed with distilled water (1:3 w/v) and then homogenized for 1 min at 8000 rpm using an Ultra-Turrax homogenizer (T18, IKA, Germany). Animal protease (Pangbo Biotech, Nanning, China) was employed for the preparation of clam protein hydrolysates under the following conditions. At enzyme/substrate ratio of 1000 U/g, the homogenate and enzymes were mixed and then incubated for 1 h at 50 °C and at pH 7.0 with stirring and then heated in a boiling-water bath for 10 min to inactivate the enzymes. Following inactivation, the content was filtered and then centrifuged, and the supernatant was dried using a vacuum freeze-dryer (Tokyo Rikakikai Co. Ltd., Japan) to obtain CPH, which were stored at −20 °C for further studies.

The degree of hydrolysis (DH) of CPH was determined as previous described (Cao et al., 2012). The amino acid analysis of CPH was performed using an automatic amino acid analyzer (Hitachi High-Technologies Co., Japan), after acid-hydrolysis with 6 M HCl at 110 °C for 22 h. Because glutamine is converted to glutamic acid and asparagine to aspartic acid during acid-hydrolysis, the values given for glutamic acid and aspartic acid include glutamine and asparagine, respectively.

2.2 Ultrafiltration of CPH

A portion of CPH was fractionated by WTM-1812G ultrafiltration (Watech Member Corporation, Hangzhou, China) with ultrafiltration membranes having molecular weight cut-off (MWCO) of 10, 5, and 2.5 kDa, respectively. CPH was passed through the 10 kDa MWCO membrane first, meaning retentate 1 (designated as CPH-1) was greater than 10 kDa, and the flow-through was less than 10 kDa fraction. The flow-through less than 10 kDa was passed through the 5 kDa MWCO membrane, meaning retentate 2 (designated as CPH-2) had an MW range between 10 and 5 kDa and the flow-through of less than 5 kDa. The less than 5 kDa fraction was passed through the 2.5 kDa MWCO membrane, meaning retentate 3 (designated as CPH-3) had an MW range between 5 and 2.5 kDa, and the final flow-through (designated as CPH-4) was less than 2.5 kDa. All recovered ultrafiltration fractions of CPHs were vacuum freeze-dried and saved for further studies.

The molecular weight (MW) distribution of CPHs was estimated by a size exclusion chromatography method using an HPLC system (Shimadzu, Japan) with a Protein-pak 60A column (7.8 mm × 300 mm, Waters, USA) according to our previous study (Li et al., 2019). A 20 μL of sample (1.0 mg/mL) was loaded on the column that was connected to the HPLC system. The sample was eluted using Tris-HCl buffer (50 mM, pH 8.3) at a flow rate of 0.7 mL/min (25 °C), and elution was monitored at 214 nm characteristic absorbance maxima for peptide bonds. Lysozyme (14,300 Da), insulin (5808 Da), thymosin α1 (3108 Da), vitamin B12 (1855 Da), and *N*-Hippuryl-His-Leu hydrate (429.47 Da) were used as molecular weight standards.

2.3 Animal treatments and experimental design

Male Kunming mice (20 ± 2 g) were supplied by the Institute of Medical Laboratory Animals, Chinese Academy of Medical Sciences (Beijing China), certificate of animal quality: SCXK (Guangdong) 2019-0204. All of the mice were housed in an environmentally controlled room at 25 ± 2 °C, relative humidity 55 ± 5%, and a 12 h light/12 h dark cycle. Tap water and basal pellet diet were supplied ad libitum to the mice. All of the animals were cared for in accordance with the standards for laboratory animals established by China (GB14925-2001) and handled according to the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals.

The treatment of animals was determined according to methods previously described (Guo et al., 2019; Liu et al., 2019; Xiao et al., 2018). The mice were randomly divided into seven groups (10 mice per group; Figure 1): control group (Normal), acute alcohol model group (Model), positive group (HWJZ), CPH-1 group (CPH-1), CPH-2 group (CPH-2), CPH-3 group (CPH-3), and CPH-4 group (CPH-4). Herein, Haiwangjinzun (HWJZ), used as a positive control, is a commercial product claimed to facilitate alcohol metabolism efficacy in the human body and contains oyster extracts, Vitamin C, L-cysteine, and

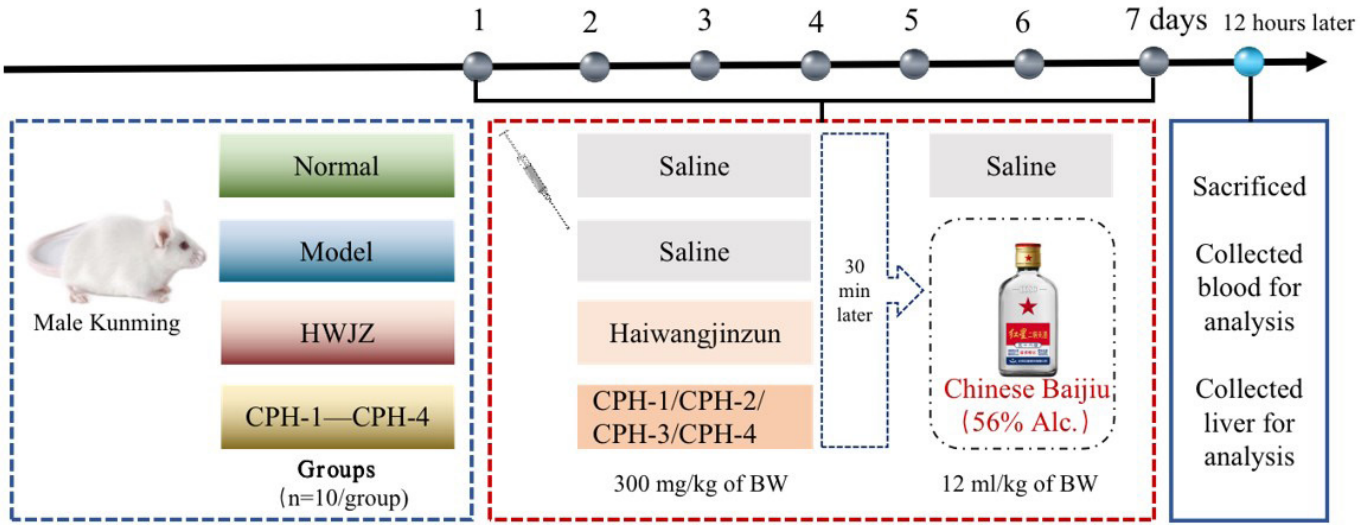

Figure 1. The flow chart of experimental design. Normal, control group; Model, acute alcohol model group; HWJZ, positive group; CPH-1—CPH-4, CPH-1 group, CPH-2 group, CPH-3 group, and CPH-4 group.

taurine. Mice were administered intragastrically with HWJZ, CPH-1, CPH-2, CPH-3, and CPH-4 at doses of 300 mg/kg bodyweight (bw) for the positive, CPH-1, CPH-2, CPH-3, and CPH-4 groups, respectively. An identical volume of saline was administered to mice in the Normal and Model groups in the same manner. Thirty minutes later, each group of mice was administered with 12 mL/kg bw Erguotou (a Chinese Baijiu, alcohol 56% v/v) to induce acute liver injury every 24 h for continuous 7 days except for the Normal group, which received an identical volume of saline instead. Body weight was measured before daily administration so that the exact dose of sample or alcohol was confirmed.

2.4 Blood collection and determination of biochemical parameters

The blood samples of the mice were collected via enucleation of eyeballs after 12 h fasting following the last alcohol treatment, and then the mice were sacrificed by cervical dislocation. The serum was isolated for assaying serum aspartate transaminase (AST) and alanine transaminase (ALT). The levels of AST and ALT in the serum samples were determined using commercial kits from Nanjing Jiancheng Co. Ltd. (Nanjing, China) according to the manufacturer's instructions.

2.5 Biochemical parameters determination and histological examination of the livers

The livers were taken out immediately and rinsed with normal saline solution after sacrifice. Five livers of each group were homogenized in an ice-cold phosphate buffer (0.05 M, pH 7.4) using a G50 tissue grinder (Coyote, Beijing, China) with disposable plastic pestle and then centrifuged at 12,000 × g and 4 °C for 10 min. The resulting supernatant was used to determine the levels of GSH, MDA, and TG, and activities of ADH and ALDH, by commercial kits from Nanjing Jiancheng Co. Ltd. (Nanjing, China), according to the manufacturer's instructions. All of the results were normalized to total protein detected using BCA kits (Dingguo Changsheng, Beijing, China) according to the manufacturer's instructions.

The other liver specimens were fixed overnight in 4% neutral buffered paraformaldehyde solution. The fixed tissues were embedded in paraffin and stained with hematoxylin and eosin (H&E). Frozen liver sections were used for Oil Red-O staining (5 μm in thickness). The stained sections were analyzed under light microscope (Olympus, Tokyo, Japan). Tissue images were taken and quantified using the Olympus CellSens Dimension software (version 2.2, Olympus, Tokyo, Japan, 2009). The severity of liver injury level was valued according to proportion of lipid droplets involved as follows: 0, lipid droplets in liver cells scattered, scarce and normal; 1, hepatocytes with lipid droplets do not exceed 1/4 of the entire picture (scattered); 2, hepatocytes with lipid droplets do not exceed 1/2 of the entire picture (scattered); 3, hepatocytes with lipid droplets do not exceed 3/4 of the entire picture (dispersed); 4, lipid droplets affected almost the entire liver tissue (filled) (Xin et al., 2019). All assessments were made on five fields per section and five sections per liver.

2.6 Purification of hepatoprotective peptide

The UF fraction with the highest hepatoprotective effect obtained previously was subjected to gel filtration HPLC on a Sephacryl S-100 column (2.6 × 60 cm, GE Healthcare Biosciences, Buckinghamshire, UK) that was equilibrated and eluted with distilled water at a flow rate of 1 mL/min. Fractions with high ADH activation rate were collected and subjected to HPLC on an analytical reverse-phase Symmetry C-18 column (250 × 4.6 mm, 5 μm, Waters); the column was equilibrated with 0.1% (v/v) TFA/water and developed by a linear gradient of acetonitrile (5-30% for 0-15 min and 30% for 20 min) with 0.1% TFA (v/v) at a flow rate of 1 mL/min. At each chromatographic step, the fractions were monitored by recording A_{214}, and the ADH activation rate was determined after removing acetonitrile and TFA under a stream of nitrogen using the ADH activation assay *in vitro*.

2.7 ADH activation in vitro

The activity of CPHs on yeast ADH (Sigma, USA) activation *in vitro* was determined by a commercial kit (Nanjing Jiancheng, Nanjing, China) with modification of the previous method (Xiao et al., 2018). Briefly, 50 μL of CPHs dispersion at specific concentration was mixed with 150 μL of detection reagent (a mixture containing NAD^+ and ethanol in buffer according to the instructions of the manufacturer). After equilibrating at 37 °C for 5 min, the reaction was initiated by addition of 50 μL ADH (0.2 U/mL). Absorbance at OD_{340} nm was recorded every 10 s for 10 min using a Varioskan Flash multimode reader (Thermo Scientific, MA, USA). Distilled water was used as a control. All the determinations were carried out in triplicates. The ADH activation rate was calculated by the following Formula 1:

$$Percentage\ of\ ADH\ activation\ (\%) = \frac{\text{Sample}(A_2\text{-}A_1)\text{-Control}(A_2\text{-}A_1)}{\text{Control}(A_2\text{-}A_1)} \times 100\% \quad (1)$$

Here, A1 is the initial reaction rate, while A2 is the reaction rate after 10 min.

2.8 Characterization of hepatoprotective peptide

The RP-HPLC fractions showing the highest ADH activation rate were further resolved by ultra-performance liquid chromatography (UPLC) and analyzed by high-resolution electrospray mass spectrometry on a Waters Xevo-G2-XS QTof (QTof LC/MS/MS). Samples were dissolved in 20% dimethyl sulfoxide and separated on a BEH C18 column (130 Å, 1.7μ, 2.1 × 50 mm, Waters) at 300 μL/min with a gradient elution of 20-80% aqueous acetonitrile + 0.1% formic acid within 30 min. MS and MS/MS spectra were collected in positive ion mode. Spectra were recorded in positive resolution mode with the following settings: capillary voltage, 2.5 kV; cone voltage, 40 V; source temperature, 120 °C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 700 L/h; desolvation temperature, 450 °C. Continuous MSE spectra were recorded in a mass range of 50-1200 *m*/*z* with a scan time of 1 s. Collision energy was ramped from 14 to 45 eV. Serving as mass reference, leucine-encephalin was injected with a capillary voltage of 3 kV every 60 s. The mass spectra were processed with ProteinLynx Global Server (Waters, version 2.5.2).

2.9 Peptide synthesis

The peptides identified by LC-MS/MS were synthesized by solid-phase peptide synthesis (China Peptides Co., Ltd.). The purity of the synthesized peptides was analyzed by HPLC. The synthesized peptides were also subjected to LC-MS/MS for MS analysis.

2.10 Statistical analysis

Data are expressed as means ± standard deviation (SD). Statistical analysis was performed using SPSS software (version17.0, SPSS Inc, USA). Statistical analysis was performed using one-way analysis of variance (ANOVA) with Duncan's new multiple-range test, and $p < 0.05$ values were considered as statistically significant.

3 Results and discussion

3.1 Preparation and characteristic of the CPH

The most common way to produce bioactive peptides is through enzymatic hydrolysis of whole protein molecules (Li et al., 2022). In our study, edible meat from *C. fluminea* was enzymatically hydrolyzed with commercial enzymes for the preparation of functional peptides, the %DH value was around 15%. The properties and biological functions of a hydrolysate are closely related to its DH, and this DH of CPH is crucial to reproduce our work.

The activity of peptides in hydrolysates is a direct consequence of their amino acid composition and sequence (Asha et al., 2016). As shown in Table 1, total amino acid analysis indicated that CPH contained a high amount of essential amino acids (45.78%). It is reported that the ingestion of amino acids could have nutritional and physiological functions for rats that consume alcohol; however, the efficiency of alcohol metabolism may be reduced in conditions of insufficient essential amino acids (Yamaguchi, 1998). In addition, the total hydrophobic amino acid content in CPH constituted 44.97%, including 8.12% of Ala, 5.62% of Pro, 8.03% of Leu and others.

The presence of some hydrophobic amino acids like Leu, Pro, and Val in peptides tends to enhance the antioxidant activity due to the increasing accessibility of hydrophobic residues to the reactive species (Zheng et al., 2016). It is also reported that the mechanism by which peptides facilitate alcohol metabolism could be associated with the presence of some specific amino acids or peptides containing such amino acids, namely Lys, Pro, Leu, and Ala (Xiao et al., 2018). CPH contained 20.88% of branched-chain amino acids, and Yamaguchi (1998) proved that corn peptides, rich in branched-chain amino acids, are capable of lowering blood alcohol concentration levels by causing

Table 1. Amino acid composition of clam (*Corbicula fluminea*) protein hydrolysate (CPH).

Amino Acids	Content	Amino Acids	Content	Amino Acids	Content	Amino Acids	Content
Glu	14.63	Pro	5.62	His	2.59	Val	7.05
Asp	10.35	Gly	6.07	Lys	8.12	Ile	5.80
Arg	3.84	Ser	4.55	Leu	8.03	Phe	4.28
Ala	8.12	Tyr	1.07	Thr	7.23	Met	2.68
EAA[a]	45.78	BCAA[b]	20.88	HAA[c]	44.97	AAA[d]	14.48

[a]EAA: essential amino acid, including Lys, Leu, Thr, Val, Ile, Phe His, and Met; [b]BCAA: branched-chain amino acids, including Leu, Ile, and Val; [c]HAA: hydrophobic amino acid, including Ala, Pro, Gly, Leu, Val, Ile, and Phe; [d]AAA: antioxidant amino acids, including Lys, His, Tyr, and Met (Lin et al., 2017).

malabsorption of ethanol through the gastrointestinal tract or delaying ethanol release from the stomach. Besides, CPH was rich in Glu and Asp, which accounted for amounts of 14.63% and 10.35%, respectively. It has been recognized that acidic amino acids such as Glu and Asp contributed to the antioxidant activities of peptides due to the presence of excess electrons that could be donated during interaction with free radicals (Cai et al., 2017; Udenigwe & Aluko, 2011). Ethanol consumption increases over-production of cellular reactive oxygen species and other prooxidants and depletes endogenous antioxidants, leading to oxidative stress in many organs, especially in the liver where ethanol is mainly metabolized to acetaldehyde (Feng et al., 2019). Hence, oxidative stress is considered to be a key risk factor in the development of hepatic diseases. CPH contained 14.48% of antioxidant amino acids, and the presence of these antioxidant amino acids, including Trp, Tyr, Cys, His, and Met, is considered to be the determining factor for radical scavenging activity (Zheng et al., 2016). There are many studies that have shown that the administration of antioxidants or antioxidant-rich extracts can prevent or ameliorate the toxic actions of ethanol (Dogan & Anuk, 2019).

3.2 Molecular weight distribution of CPHs

Besides amino acid composition, the MW of peptides is also a significant factor that reflects the biological activities of peptides. The MW distribution of CPH was evaluated using high-performance liquid chromatography (HPLC), and the results are shown in Table 2. The MW of 20.47% of the peptides in CPH was less than 2.5 kDa, that of 13.26% was 2.5-5 kDa, while those of 41.82% and 24.45% were 5-10 kDa and more than 10 kDa, respectively. These were as expected, CPH contained large amount (66.2%) of high MW (>5 kDa) peptides. The fact attributed to the relatively low DH (15.27 ± 2.23%) of CPH, compared with the DH of oyster *Crassostrea hongkongensis* hydrolysate (27.97%) (Li et al., 2019) and of clam *Paphia undulata* hydrolysate (27.25-36.23%) (He et al., 2015). DH exert an impact on the MW distribution of hydrolysate, further degradation is better for generating more small peptides and free amino acids. At any rate, the purpose of this study was to screen the unknown bioactive peptides from the hydrolysates; furthermore, low DH is sufficient for the liberation of bioactive peptides (Gómez Sampedro & Zapata Montoya, 2014).

In order to obtain a peptide population enriched in a selection of sizes, successive ultrafiltration was used to refine and to fractionate CPH in our study. As shown in Table 2, the MWs of different ultrafiltration fractions were distributed in each MWCO region: the > 10 kDa and 5-10 kDa fractions accounted for 43% and 29% of CPH-1, respectively; the 5-10 kDa fraction accounted for 42% of CPH-2; the 2.5-5 kDa fraction accounted for 41% of CPH-3, while the < 2.5 kDa fractions accounted for 32% and 50% of CPH-3 and CPH-4, respectively. This result was consistent with previous studies, which suggests that ultrafiltration was effective in removing large and medium MW organic matter but was ineffective in removing low MW matter (Zha et al., 2016).

Table 2. Molecular weight distribution of clam (*Corbicula fluminea*) protein hydrolysates (CPHs) (%).

	<2.5 kDa	2.5-5 kDa	5-10 kDa	>10 kDa
CPH	20.47	13.26	41.82	24.45
CPH-1	13.34	14.15	29.41	43.09
CPH-2	20.13	20.53	42.25	17.09
CPH-3	32.29	41.65	15.13	10.93
CPH-4	50.26	21.39	19.12	9.23

3.3 Effect of CPHs on alcohol-induced liver injury in mice

The activities of AST and ALT are commonly identified as major indexes for diagnosing hepatic injury, especially AST leakage for relatively serious liver injury, which can reflect mitochondrial damage (Zhao et al., 2019). The effects of CPHs on mice AST and ALT levels are shown in Table 3. In comparison with the normal group, alcohol treatment significantly elevated ($p < 0.05$) the plasma AST and ALT levels by proximately 42.2% and 126.8% from 11.913 to 16.945 U/L and 6.815 to 15.456 U/L, respectively. Accumulating evidence has demonstrated that alcohol-induced AST and ALT activities in serum were elevated during alcoholic liver disease (Das et al., 2018; Xiao et al., 2018). This indicated that the alcohol-induced liver injury model in mice was well-established in this study. In comparison with the Model group, mice AST levels in CPH-1 to 4 groups decreased by 10.3%, 11.35%, 16.1%, and 9.6%, respectively. ALT levels decreased by 25.8%, 38.4%, 45.0%, and 29.6%, respectively. Especially when CPH-3 was applied, serum ALT and AST levels were close to those of the Normal group, and the protective effect is more noticeable than in the HWJZ group, suggesting that CPHs can enhance cytomembrane stability, thereby repairing the alcohol-damaged liver function.

Alcohol metabolism mainly occurs through two pathways including the ADH oxidation system and the microsome alcohol oxidation system. The ADH dominated pathway is considered the most important, through which about 90% of alcohol is transformed into acetaldehyde and acetic acid by ADH and aldehyde dehydrogenase (ALDH), respectively. Meanwhile, an increasing amount of evidence has accumulated showing that alteration of liver ADH activity can lead to corresponding changes in the rate of alcohol metabolism, further supporting the idea that the level of liver ADH activity is a key factor governing the rate of alcohol metabolism *in vivo*. The beneficial effect of CPHs on alcohol deposition was confirmed using ADH and ALDH enzyme assay. As for the changes in ADH, acute alcohol ingestion significantly suppressed the ADH and ALDH activities by over 18% and 32%, respectively, as compared with the Normal group (Table 3). Administration of CPHs could significantly reduce ($p < 0.05$) the suppression. Specifically, when compared to the Model group, CPH-1 and CPH-4 groups showed no protective effect, while CPH-2 and CPH-3 administration could remarkably increase ADH and ALDH activities, even higher than the HWJZ group ($p < 0.05$).

The hepatic levels of malondialdehyde (MDA) and glutathione (GSH) are considered as typical indexes of antioxidant status of tissues (Jiang et al., 2019). GSH is the major non-protein thiol that plays a vital role in maintaining the antioxidant defense mechanism in the body (Guldas et al., 2021). MDA, the end

Table 3. Effects of CPHaps on the GSH, MDA, and TG levels, the activities of ADH and ALDH in liver, and the ALT and AST levels in serum of alcohol-induced acute liver injury mice.

Groups	Normal	Model	HWJZ	CPH-1	CPH-2	CPH-3	CPH-4
ALT (U/L)	6.815 ± 1.394^{e}	15.456 ± 1.133^{a}	14.977 ± 0.901^{a}	11.451 ± 0.786^{b}	9.514 ± 1.067^{cd}	8.494 ± 0.761^{de}	10.871 ± 0.924^{bc}
AST (U/L)	11.913±0.584^{c}	16.945 ± 0.597^{a}	14.425 ± 1.889^{b}	15.195 ± 0.540^{ab}	15.021 ± 0.261^{ab}	14.222 ± 0.423^{b}	15.320 ± 1.813^{ab}
ADH (U/mg protein)	1.717 ± 0.070^{b}	1.399 ± 0.156^{c}	1.540 ± 0.177b^{c}	1.358 ± 0.150^{c}	1.992 ± 0.205^{a}	1.703 ± 0.158^{b}	1.313 ± 0.278^{c}
ALDH (U/mg protein)	9.680 ±1.985^{ab}	6.517 ± 0.553^{cd}	5.464 ± 0.452^{d}	7.453 ± 2.878^{bcd}	10.830 ± 1.225^{ab}	11.744 ± 2.228^{a}	8.920 ± 2.501^{abc}
MDA (nmol/mg protein)	1.331 ± 0.051^{e}	1.981 ± 0.064^{a}	1.597 ± 0.024^{c}	1.676 ± 0.026^{b}	1.476 ± 0.063^{d}	1.422 ± 0.032^{d}	1.701 ± 0.022^{b}
TG (nmol/mg protein)	70.089 ± 5.134^{c}	92.211 ± 7.870^{a}	72.737 ± 4.090^{c}	80.192 ± 3.381^{b}	79.476 ± 4.759^{b}	79.992 ± 5.760^{b}	80.795 ± 1.246^{b}
GSH (nmol/mg protein)	0.161 ± 0.006^{a}	0.112 ± 0.005^{e}	0.126 ± 0.008^{d}	0.129 ± 0.006^{d}	0.140 ± 0.004^{c}	0.151 ± 0.005^{b}	0.143 ± 0.006^{c}

Different letters (a-e) in the same line denote significant differences (P< 0.05).

product of lipid peroxidation, is widely used as a marker of lipid peroxidation injury. Alcohol metabolism with oxidative stress damage results in the formation of MDA that can bind to proteins to form adducts. Therefore, the MDA level and activity of GSH in the mouse liver were measured in the present work. As shown in Table 3, MDA level in alcohol-treated mice was about 1.49-fold higher than that of the untreated normal mice ($p < 0.05$). Additionally, the distinct decrease of GSH activities was found in liver homogenate of alcohol-induced mice as compared to the Normal group, indicating that the mice treated with alcohol suffered from oxidative stress. However, reductions of these parameters were significantly ameliorated by treatment with CPHs or HWJZ when compared with the Model group. The activity of GSH reached the maximum in the CPH-3 group, which was 34.82% higher than that in the Model group. Meanwhile, MDA level was significantly decreased from 1.981 to 1.422 nmol/mg prot in liver tissues obtained from the CPH-3 group; it was similar to that of the Normal group (1.331 nmol/mg prot) but had a significant difference. Noticeably, CPH-3 was more effective than HWJZ in regulating MDA and GSH activities, suggesting a potential to protect the liver against high oxidative stress induced by acute alcohol ingestion.

Formation of liver lipid is the earliest and most common response to excessive drinking. To evaluate liver damage induced by alcohol, hepatic triglyceride (TG) level was also determined in our work. As shown in Table 3, hepatic TG level in the Model group was higher ($p < 0.05$) than that of the Normal group, while that level was reduced significantly in the CPHs- and HWJZ-treatment groups, indicating that CPHs can lower the risk of alcohol-induced liver fat formation. The representative photomicrographs of liver sections from the experimental groups were shown in Figure 2A. The pathology section examination from the normal control group showed that the liver tissue organization is regular and the hepatic lobules of them was clear without liquid droplets, liver sinusoid express normal, and the hepatic cord was well-arranged. However, the photomicrographs of liver sections from the model groups which treated by ethanol demonstrated that cytoplasm loosening of hepatic cells, diffuse ballooning degeneration and microvesicular steatosis, lipid degeneration, diffuse ballooning degeneration led to disrupted liver lobule structure. Moreover, there were acidophilic and necrotic liver cells in the photomicrograph. Granular degeneration could be found in some liver cells. And the inflammatory cells infiltrated were observed in the necrotic lesion. Conversely, the groups dealt with HWJZ and CPHs significantly reversed the ethanol-induced liver injury. The liver tissue displayed an intact architecture, and only a few liver cells had ballooning and steatosis, and hepatocyte regeneration and no necrotic cells were observed. Moreover, a quantitative evaluation (steatosis score) also showed that the lipid accumulation area of the CPHs or HWJZ groups were less ($p<0.05$) than alcoholic induced mice without any supplementation (Model group), especially CPH-3 group ($p<0.05$) (Figure 2B).Taken together, the fact that CPH-3 exhibited potent hepatoprotective effect against alcohol-induced liver injury in mice made this fraction an excellent source for identifying hepatoprotective peptides. Taken together, the fact that CPH-3 exhibited potent hepatoprotective effect against alcohol-induced liver injury in mice made this fraction an excellent source for identifying hepatoprotective peptides.

3.4 Purification of hepatoprotective peptide

For the identification of hepatoprotective peptide from CPH, gel filtration column chromatography was conducted. CPH was dissolved in distilled water and loaded on a Sephacryl S-100 column at a flow rate of 1.0 mL/min. The elution peaks were monitored at 214 nm, and peptide fractions were divided into four fractions, which were labelled as F1, 2, 3, and 4 (Figure 3A). Each peak was pooled and evaluated for ADH activation.

Research work on ALD therapies has commonly used *in vivo* models; however, the high cost, time consumption, and complicated nature of experiments using this method limit its application in sample screening. Thus, researchers have increasingly been using the alcohol dehydrogenase (ADH) activation assay *in vitro* to evaluate the effect of samples on alcohol metabolism. This can provide an indication of ADH activity *in vivo* (Xiao et al., 2018; Yu et al., 2013), which is the major rate-limiting factor in alcohol metabolism. Consequently, in this article, the activity of CPHs on yeast ADH activation *in vitro* was studied for sample screening.

As shown in Figure 3B, F2 exhibited 36.37% ADH activation at 0.5 mg/mL, followed by F1 (30.53%), which were both higher than HWJZ ($p <0.05$). Thus, fraction F2 was lyophilized and further purified using reverse phase HPLC (RP-HPLC) on a

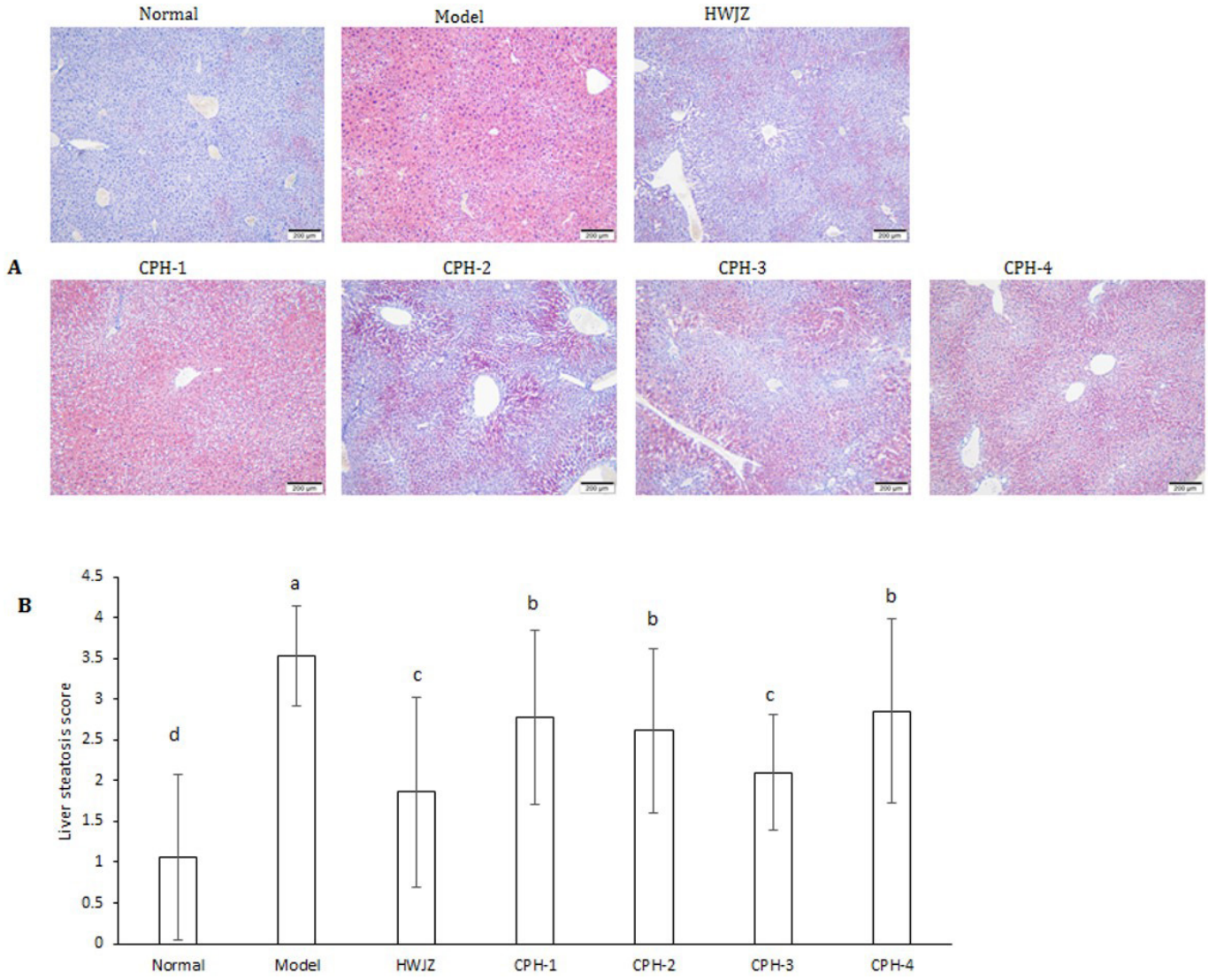

Figure 2. Effects of alcohol, Haiwangjinzun (HWJZ) and the clam protein hydrolysates (CPHs) on hepatic histological changes in liver tissue of mice (Hematoxylin and eosin, 100 ×, Scale bars = 200 μm) (A) and liver steatosis score (B). Values are means ± SD of three determinations. Bars with different letters are significantly different (P < 0.05).

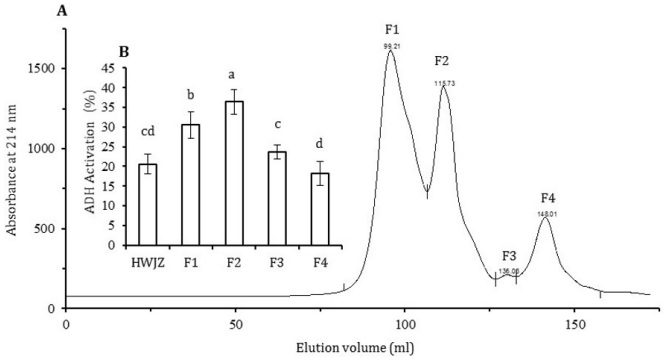

Figure 3. Gel filtration chromatography of CPH-3 on a Sephacryl S-100 column (2.6 × 60 cm) with distilled water at a flow rate of 1 mL/min (A). Four fractions (F1, F2, F3, and F4) were separated, and their ADH activation values *in vitro* were evaluated at 0.5 mg/mL (B). Values are means ± SD of three determinations. Bars with different letters are significantly different ($p < 0.05$).

Symmetry C-18 column with a linear gradient of acetonitrile with 0.1% trifluoroacetic acid (TFA) (v/v) at a flow rate of 1 mL/min. Seven peaks were seen with absorption maxima at 214 nm characteristic for peptide bonds (Figure 4A). ADH activation rate of the seven fractions pooled from RP-HPLC runs was tested by ADH activation assay *in vitro*, and the results are shown in Figure 4B. Fraction F24 possessed the highest ADH activation value with 26.36% at 0.2 mg/mL, followed by fraction F22 showing 23.80%, both higher than that of HWJZ showing 17.01% at 0.5 mg/mL. Therefore, the peptide fraction F24 was further analyzed.

3.5 Identification of hepatoprotective peptide by LC-MS/MS

Since fraction F24 showed the maximum ADH activation value, the fraction was further purified using the same C18 column with a linear gradient of acetonitrile (5-30% for 0-15 min and 30% for 20 min) containing 0.1% TFA at a flow rate of 1.0 mL/min.

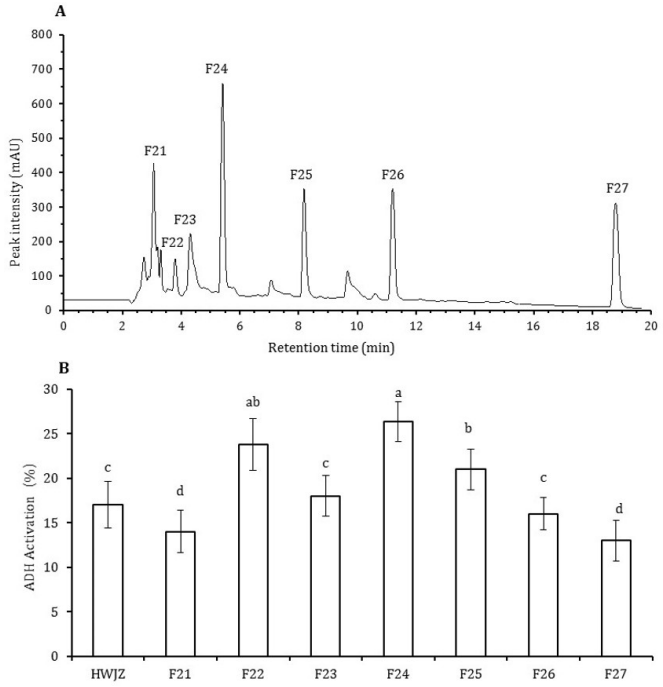

Figure 4. Reverse phase HPLC purification of F2 obtained from Sephacryl S-100 gel filtration on a Symmetry C-18 column (250 × 4.6 mm, 5 µm) with a linear gradient of acetonitrile (5-30% for 0-15 min and 30% for 20 min) with 0.1% TFA (v/v) at a flow rate of 1 mL/min (A). The eluted peaks were collected, and their ADH activation values *in vitro* were evaluated at 0.2 mg/mL, while *in vitro* ADH activation values of HWJZ were evaluated at 0.5 mg/mL (B). Values are means ± SD of three determinations. Bars with different letters are significantly different ($p < 0.05$).

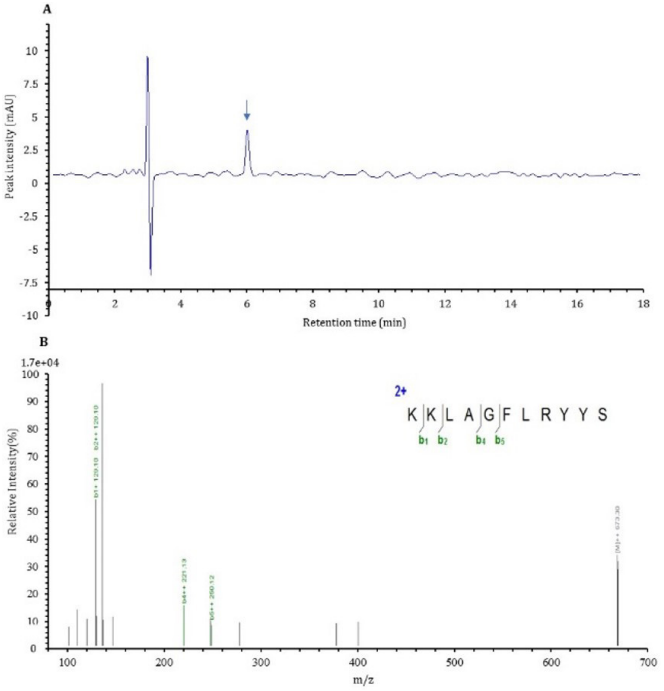

Figure 5. Peptide identification from the F24 fraction via liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF/MS/MS). (A) The reverse phase HPLC chromatogram of the F24 fraction before identification by MS/MS. Peptide, KKLAGFLRYYS was identified from the F24 fraction (B).

As shown in Figure 5A, fraction F24 was not further separated into other fractions, and it exerted potent ADH activation activity. This fraction was analyzed for amino acid sequence using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF/MS/MS). As shown in Figure 5B, the hepatoprotective peptide identified from the 2.5-5 kDa peptide fraction obtained from *C. fluminea* by animal protease hydrolysis was determined to be Lys-Lys-Leu-Ala-Gly-Phe-Leu-Pro-Tyr-Tyr-Ser. Its theoretical molecular mass is 1330.68 Da, and the hydrophobicity was determined to be about 33.3% using online peptide analysis tools (Peptide 2.0 Inc., 2022). Identified hepatoprotective peptide was chemically synthesized by solid-phase synthesis (purity 97.87%), and its ADH activation activity *in vitro* was also evaluated: ADH activation values of 14.63% and 34.17% at 50 and 100 µg/mL, respectively.

Park et al. (2016) purified and identified three antioxidant peptides from blue mussel (*Mytilus edulis*) hydrolysate; two of them, Pro-Ile-Ile-Val-Tyr-Trp-Lys (1004.57 Da) and Phe-Ser-Val-Val-Pro-Ser-Pro-Lys (860.09 Da), exhibited hepatoprotective effects against oxidative stress-induced hepatotoxicity in cultured hepatocytes. Ma et al. (2012) identified a peptide, Gln-Leu-Leu-Pro-Phe (639.7 Da), from hydrolysate of corn protein, which showed an effect of eliminating alcohol. Asha et al. (2016) reported an antioxidant peptide Ile-Ser-Ile-Gly-Gly-Gln-Pro-Ala-Gly-Arg-Ile-Val-Met (1298 Da) obtained from oyster protein hydrolysate; and Ngo et al. (2010) identified antioxidant peptide Asp-Pro-Ala-Leu-Ala-Thr-Glu-Pro-Asp-Pro-Met-Pro-Phe (1382.57 Da) from tilapia scale. Collectively, low molecular weight (below 1500 Da) hepatoprotective or antioxidant peptides from different organisms have been documented, and in the present study, we also identified one hepatoprotective peptide with below 1500 Da molecular weight. Specifically, the peptide we identified contains four antioxidant amino acids: two Lys and two Tyr. There are many studies that have shown that the administration of antioxidants or antioxidant-rich extracts can prevent or ameliorate the toxic actions of ethanol (Dogan & Anuk, 2019). Besides, this peptide contains Ala and two Leu, and it is reported that peptides containing specific amino acids Lys, Pro, Leu, and Ala might facilitate alcohol metabolism (Park et al., 2016; Udenigwe & Aluko, 2011; Xiao et al., 2018).

4 Conclusions

Our study showed that CPH-3 exhibited hepatoprotective effects in acute alcoholic liver injury in mice. One of the seven fractions (F24) obtained by RP-HPLC that showed the good hepatoprotective property *in vitro* was subjected to LC-MS/MS, and its sequence was determined as KKLAGFLRYYS (1330.68 Da). Results suggested the peptide fraction CPH-3 and/or hepatoprotective peptide KKLAGFLRYYS may be considered for use as an ingredient in new functional foods. However, further studies are needed to prove *in vivo* hepatoprotective activity in order to find practical usage of the peptide.

Abbreviations

ADH: alcohol dehydrogenase; ALD: alcoholic liver disease; CPH(s): clam protein hydrolysate (s); AST: serum aspartate transaminase; ALT: alanine transaminase; MDA: malondialdehyde; TG: triglyceride; ALDH: aldehyde dehydrogenase; GSH: glutathione; FCW: hot-water extract of freshwater clam; DH: degree of hydrolysis; MWCO: molecular weight cut-off; HWJZ: Haiwangjinzun.

Ethical approval

This study was carried out in strict accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of the Chinese Association for Laboratory Animal Science. All animal care and protocols were approved by the Animal Experimentation Ethics Committee of the Guangdong Ocean University.

Conflict of interes

There are no conflicts to declare.

Author contributions

Methodology, Jialong Gao and Chaohua Zhang; validation, Jialong Gao and Wenhong Cao; formal analysis, Jialong Gao and Wenhong Cao; investigation, Jialong Gao, Zhongqin Chen and Jianping Chen; resources, Wenhong Cao, Huina Zheng and Haisheng Lin; data curation, Jialong Gao and Chaohua Zhang; writing—original draft preparation, Jialong Gao; writing—review and editing, Jialong Gao; supervision, Chaohua Zhang and Yujin Li; project administration, Chaohua Zhang and Xiaoming Qin. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

The program was supported by the Guangdong Province Innovation School Project (2020KZDZX1112) and the special funds for the Construction of Modern Agricultural Industrial Technology System (No. CRS-49).

References

Asha, K. K., Remya Kumari, K. R., Ashok Kumar, K., Chatterjee, N. S., Anandan, R., & Mathew, S. (2016). Sequence determination of an antioxidant peptide obtained by enzymatic hydrolysis of oyster *Crassostrea madrasensis* (Preston). *International Journal of Peptide Research and Therapeutics*, 22(3), 421-433. http://dx.doi.org/10.1007/s10989-016-9521-0.

Bai, J., Chen, Y., Ning, Z., Liu, S., Xu, C., & Yan, J. K. (2020). Proteoglycan isolated from *Corbicula fluminea* exerts hepato-protective effects against alcohol-induced liver injury in mice. *International Journal of Biological Macromolecules*, 142, 1-10. http://dx.doi.org/10.1016/j.ijbiomac.2019.12.001. PMid:31805321.

Cai, X., Yan, A., Fu, N., & Wang, S. (2017). In vitro antioxidant activities of enzymatic hydrolysate from *Schizochytrium* sp. and its hepatoprotective effects on acute alcohol-induced liver injury in vivo. *Marine Drugs*, 15(4), 115. http://dx.doi.org/10.3390/md15040115. PMid:28394291.

Cao, W., Zhang, C., Ji, H., & Hao, J. (2012). Optimization of peptic hydrolysis parameters for the production of angiotensin I-converting enzyme inhibitory hydrolysate from Acetes chinensis through Plackett-Burman and response surface methodological approaches. *Journal of the Science of Food and Agriculture*, 92(1), 42-48. http://dx.doi.org/10.1002/jsfa.4538. PMid:21732383.

Chen, X., Fang, F., & Wang, S. (2020). Physicochemical properties and hepatoprotective effects of glycated Snapper fish scale peptides conjugated with xylose via maillard reaction. *Food and Chemical Toxicology*, 137, 111115. http://dx.doi.org/10.1016/j.fct.2020.111115. PMid:31911128.

Chijimatsu, T., Umeki, M., Kobayashi, S., Kataoka, Y., Yamada, K., Oda, H., & Mochizuki, S. (2015). Dietary freshwater clam (*Corbicula fluminea*) extract suppresses accumulation of hepatic lipids and increases in serum cholesterol and aminotransferase activities induced by dietary chloretone in rats. *Bioscience, Biotechnology, and Biochemistry*, 79(7), 1155-1163. http://dx.doi.org/10.1080/09168451.2015.1012147. PMid:25704646.

Das, M., Basu, S., Banerjee, B., Sen, A., Jana, K., & Datta, G. (2018). Hepatoprotective effects of green *Capsicum annum* against ethanol induced oxidative stress, inflammation and apoptosis in rats. *Journal of Ethnopharmacology*, 227, 69-81. http://dx.doi.org/10.1016/j.jep.2018.08.019. PMid:30118838.

Dogan, A., & Anuk, O. O. (2019). Investigation of the phytochemical composition and antioxidant properties of chinar (*Platanus orientalis* L.) leaf infusion against ethanol-induced oxidative stress in rats. *Molecular Biology Reports*, 46(3), 3049-3061. http://dx.doi.org/10.1007/s11033-019-04741-7. PMid:30864112.

Feng, R., Chen, J. H., Liu, C. H., Xia, F. B., Xiao, Z., Zhang, X., & Wan, J. B. (2019). A combination of *Pueraria lobata* and *Silybum marianum* protects against alcoholic liver disease in mice. *Phytomedicine*, 58, 152824. http://dx.doi.org/10.1016/j.phymed.2019.152824. PMid:30836218.

Gómez Sampedro, L. J., & Zapata Montoya, J. E. (2014). Effects of hydrolysis and digestion in vitro on the activity of bovine plasma hydrolysates as inhibitors of the angiotensin I converting enzyme. *Brazilian Archives of Biology and Technology*, 57(3), 386-393. http://dx.doi.org/10.1590/S1516-89132014005000004.

Guldas, M., Ziyanok-Demirtas, S., Sahan, Y., Yildiz, E., & Gurbuz, O. (2021). Antioxidant and anti-diabetic properties of *Spirulina platensis* produced in Turkey. *Food Science and Technology*, 41(3), 615-625. http://dx.doi.org/10.1590/fst.23920.

Guo, P., Deng, Q., & Lu, Q. (2019). Anti-alcoholic effects of honeys from different floral origins and their correlation with honey chemical compositions. *Food Chemistry*, 286, 608-615. http://dx.doi.org/10.1016/j.foodchem.2019.02.058. PMid:30827653.

He, X. Q., Cao, W. H., Pan, G. K., Yang, L., & Zhang, C. H. (2015). Enzymatic hydrolysis optimization of *Paphia undulata* and lymphocyte proliferation activity of the isolated peptide fractions. *Journal of the Science of Food and Agriculture*, 95(7), 1544-1553. http://dx.doi.org/10.1002/jsfa.6859. PMid:25087732.

Hsu, C. L., Hsu, C. C., & Yen, G. C. (2010). Hepatoprotection by freshwater clam extract against CCl4-induced hepatic damage in rats. *The American Journal of Chinese Medicine*, 38(5), 881-894. http://dx.doi.org/10.1142/S0192415X10008329. PMid:20821820.

Huang, K. C., Wu, W. T., Yang, F. L., Chiu, Y. H., Peng, T. C., Hsu, B. G., Liao, K. W., & Lee, R. P. (2013). Effects of freshwater clam extract supplementation on time to exhaustion, muscle damage, pro/anti-inflammatory cytokines, and liver injury in rats after exhaustive exercise. *Molecules*, 18(4), 3825-3838. http://dx.doi.org/10.3390/molecules18043825. PMid:23531600.

Jiang, X., Lin, D., Shao, H., & Yang, X. (2019). Antioxidant properties of Komagataeibacter hansenii CGMCC 3917 and its ameliorative effects on alcohol-induced liver injury in mice. *CYTA: Journal of Food*, 17(1), 355-364. http://dx.doi.org/10.1080/19476337.2019.1584647.

Li, M., Zhang, Y., Lu, Q., Gao, Y., Ye, T., Wang, C., & Xing, D. (2022). Structure, bioactivities and applications of the polysaccharides from Tricholoma Matsutake: a review. *Food Science and Technol*, 42, e44922. http://dx.doi.org/10.1590/fst.44922.

Li, W., Xu, C., Zhang, C., Cao, W., Qin, X., Gao, J., & Zheng, H. (2019). The purification and identification of immunoregulatory peptides from oyster (Crassostrea hongkongensis) enzymatic hydrolysate. *RSC Advances*, 9(56), 32854-32863. http://dx.doi.org/10.1039/C9RA04255E. PMid:35529727.

Lin, B., Zhang, F., Yu, Y., Jiang, Q., Zhang, Z., Wang, J., & Li, Y. (2012). Marine collagen peptides protect against early alcoholic liver injury in rats. *British Journal of Nutrition*, 107(8), 1160-1166. http://dx.doi.org/10.1017/S0007114511004211. PMid:21899801.

Lin, J. J., Liu, Y. C., Chang, C. J., Pan, M. H., Lee, M. F., & Pan, B. S. (2018). Hepatoprotective mechanism of freshwater clam extract alleviates non-alcoholic fatty liver disease, elucidated in vitro and in vivo models. *Food & Function*, 9(12), 6315-6325. http://dx.doi.org/10.1039/C8FO01758A. PMid:30411763.

Lin, Y. L., Tai, S. Y., Chen, J. W., Chou, C. H., Fu, S. G., & Chen, Y. C. (2017). Ameliorative effects of pepsin-digested chicken liver hydrolysates on development of alcoholic fatty livers in mice. *Food & Function*, 8(5), 1763-1774. http://dx.doi.org/10.1039/C7FO00123A. PMid:28426073.

Liu, X., Hou, R., Yan, J., Xu, K., Wu, X., Lin, W., Zheng, M., & Fu, J. (2019). Purification and characterization of Inonotus hispidus exopolysaccharide and its protective effect on acute alcoholic liver injury in mice. *International Journal of Biological Macromolecules*, 129, 41-49. http://dx.doi.org/10.1016/j.ijbiomac.2019.02.011. PMid:30731164.

Ma, Z. L., Zhang, W. J., Yu, G. C., He, H., & Zhang, Y. (2012). The primary structure identification of a corn peptide facilitating alcohol metabolism by HPLC-MS/MS. *Peptides*, 37(1), 138-143. http://dx.doi.org/10.1016/j.peptides.2012.07.004. PMid:22789607.

Ngo, D.-H., Qian, Z.-J., Ryu, B., Park, J. W., & Kim, S.-K. (2010). In vitro antioxidant activity of a peptide isolated from Nile tilapia (*Oreochromis niloticus*) scale gelatin in free radical-mediated oxidative systems. *Journal of Functional Foods*, 2(2), 107-117. http://dx.doi.org/10.1016/j.jff.2010.02.001.

Park, S. Y., Fernando, I. P. S., Han, E. J., Kim, M. J., Jung, K., Kang, D. S., Ahn, C. B., & Ahn, G. (2019). In vivo hepatoprotective effects of a peptide fraction from krill protein hydrolysates against alcohol-induced oxidative damage. *Marine Drugs*, 17(12), 690. http://dx.doi.org/10.3390/md17120690. PMid:31817914.

Park, S. Y., Kim, Y. S., Ahn, C. B., & Je, J. Y. (2016). Partial purification and identification of three antioxidant peptides with hepatoprotective effects from blue mussel (*Mytilus edulis*) hydrolysate by peptic hydrolysis. *Journal of Functional Foods*, 20, 88-95. http://dx.doi.org/10.1016/j.jff.2015.10.023.

Peptide 2.0 Inc. (2022). Retrieved from http://peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php

Shori, A. B., Yong, Y. S., & Baba, A. S. (2021). Effects of herbal yogurt with fish collagen on bioactive peptides with angiotensin-I converting enzyme inhibitory activity. *Food Science and Technology*, 41(4), 902-907. http://dx.doi.org/10.1590/fst.24020.

Toldrá, F., Reig, M., Aristoy, M. C., & Mora, L. (2018). Generation of bioactive peptides during food processing. *Food Chemistry*, 267, 395-404. http://dx.doi.org/10.1016/j.foodchem.2017.06.119. PMid:29934183.

Udenigwe, C. C., & Aluko, R. E. (2011). Chemometric analysis of the amino acid requirements of antioxidant food protein hydrolysates. *International Journal of Molecular Sciences*, 12(5), 3148-3161. http://dx.doi.org/10.3390/ijms12053148. PMid:21686175.

Wang, X. Y., Luo, J. P., Chen, R., Zha, X. Q., & Wang, H. (2014). The effects of daily supplementation of *Dendrobium huoshanense* polysaccharide on ethanol-induced subacute liver injury in mice by proteomic analysis. *Food & Function*, 5(9), 2020-2035. http://dx.doi.org/10.1039/C3FO60629E. PMid:24933018.

Wang, Y. Y., Zhu, J., Ma, H., Ding, Z. C., Li, L., & Yan, J. K. (2019). Antidiabetic activity of a polysaccharide-protein complex from Asian Clam (*Corbicula fluminea*) in streptozotoxin-induced diabetic rats and its underlying mechanism. *Food & Function*, 10(9), 5574-5586. http://dx.doi.org/10.1039/C9FO01341E. PMid:31432066.

World Health Organization – WHO. (2018). *Global status report on alcohol and health 2018*. Geneva: WHO. Retrieved from https://www.who.int/substance_abuse/publications/global_alcohol_report/en/

Xiao, C., Zhou, F., Zhao, M., Su, G., & Sun, B. (2018). Chicken breast muscle hydrolysates ameliorate acute alcohol-induced liver injury in mice through alcohol dehydrogenase (ADH) activation and oxidative stress reduction. *Food & Function*, 9(2), 774-784. http://dx.doi.org/10.1039/C7FO01387F. PMid:29309081.

Xin, S., Wang, Z., Hao, W.-N., Yan, X.-T., Xu, Q., Liu, Y., Liu, W., Li, Y.-F., Li, X.-D., & Li, W. (2019). Liver protection effect of steamed codonopsis lanceolata on alcohol-induced liver injury in mice and its main components by LC/MS analysis. *International Journal of Pharmacology*, 15(3), 394-402. http://dx.doi.org/10.3923/ijp.2019.394.402.

Yamaguchi, M. N. F. (1998). Water soluble vegetable oligopep-tides: comparative study on alcohol metabolism and plasma amino acid concentrations in stroke-prone spontaneously hyper-tensive rats. *Food & Function*, 22, 227-244.

Yao, H. T., Lee, P. F., Lii, C. K., Liu, Y. T., & Chen, S. H. (2018). Freshwater clam extract reduces liver injury by lowering cholesterol accumulation, improving dysregulated cholesterol synthesis and alleviating inflammation in high-fat, high-cholesterol and cholic acid diet-induced steatohepatitis in mice. *Food & Function*, 9(9), 4876-4887. http://dx.doi.org/10.1039/C8FO00851E. PMid:30160281.

Yu, G.-C., Li, J.-T., He, H. U. I., Huang, W.-H., & Zhang, W.-J. (2013). Ultrafiltration Preparation of Potent Bioactive Corn Peptide as Alcohol Metabolism Stimulatorin Vivoand Study on Its Mechanism of Action. *Journal of Food Biochemistry*, 37(2), 161-167. http://dx.doi.org/10.1111/j.1745-4514.2011.00613.x.

Zha, X. S., Ma, L. M., Wu, J., & Liu, Y. (2016). The removal of organic precursors of DBPs during three advanced water treatment processes including ultrafiltration, biofiltration, and ozonation. *Environmental Science and Pollution Research International*, 23(16), 16641-16652. http://dx.doi.org/10.1007/s11356-016-6643-z. PMid:27180835.

Zhao, H., Li, H., Lai, Q., Yang, Q., Dong, Y., Liu, X., Wang, W., Zhang, J., & Jia, L. (2019). Antioxidant and hepatoprotective activities of modified polysaccharides from Coprinus comatus in mice with alcohol-induced liver injury. *International Journal of Biological Macromolecules*, 127, 476-485. http://dx.doi.org/10.1016/j.ijbiomac.2019.01.067. PMid:30659878.

Zheng, L., Zhao, Y., Dong, H., Su, G., & Zhao, M. (2016). Structure-activity relationship of antioxidant dipeptides: dominant role of Tyr, Trp, Cys and Met residues. *Journal of Functional Foods*, 21, 485-496. http://dx.doi.org/10.1016/j.jff.2015.12.003.