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RESEARCH PAPER

Antioxidant Peptide Purified from Enzymatic Hydrolysates of *Isochrysis Zhanjiangensis* and Its Protective Effect against Ethanol Induced Oxidative Stress of HepG2 Cells

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Abstract Marine microalgae have been widely applied in cosmaceuticals, nutraceuticals, and functional foods. In the present study, we first investigated the hepatoprotective effects of peptide purified from microalgae, *Isochrysis zhanjiangensis* on HepG2 cells alcoholic injury. *I. zhanjiangensis* was hydrolyzed utilizing chymotrypsin, trypsin, pepsin, and by *vitro* gastrointestinal digestion. Among hydrolysates, the gastrointestinal hydrolysate showed relatively high free radical scavenging ability preliminarily and was purified with following sequential chromatography methods. The amino acid sequence and molecular mass of the purified peptide from *I. zhanjiangensis* (PIZ) was identified as Asn-Asp-Ala-Glu-Tyr-Gly-Ile-Cys-Gly-Phe (NDAEYGICGF; MW, 1088.16 Da) via Q-TOF ESI/MS. Additionally, PIZ attenuated ethanol-induced cytotoxicity and inhibited the production of intracellular reactive oxygen species by DCFH-DA assay. Western blot results showed that superoxide dismutase (SOD) and glutathione (GSH) levels up-regulated with PIZ treatment before alcohol exposure while gamma-glutamyltransferase (GGT) protein expression down-regulated. These results provide an opportunity to discover new highly active peptide against alcohol toxicity in HepG2 cells.

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1. Introduction

Excessive and prolonged alcohol intake is considered to be associated with various problems causing physical unfitness [1]. Among them, alcoholic liver disease (ALD) is a common consequence and has been deemed as a complicated pathological process which can result in steatosis, steatohepatitis, fibrosis, and finally even cirrhosis [2]. It has been well-documented that excessive alcohol consumption can increase the production of reactive oxygen species (ROS; *e.g.* superoxide anion, hydrogen peroxide, and hydroxyl radical) and decreases the cellular antioxidant levels, leading to ALD, which has a relationship with oxidative stress [3,4]. Hence, it is a pivotal strategy to maintain the balance between ROS and systemic response in the progression of ALD. Antioxidants *in vivo*, such as superoxide dismutase (SOD) and glutathione (GSH), play an important part in alleviating the oxidative stress [5]. Gamma-glutamyltransferase (GGT) relative to redox can maintain cellular GSH metabolism and homeostasis [6]. Studies have suggested that many compounds (tyrosol, s-adenosylmethionine, chlorogenic acid, and erythromycin) may be useful in the treatment of ALD, but they have some side effects (neurotoxicity and liver damage) and lack of scientific evidence [7-10]. Therefore, seeking a synthetic or natural antioxidant scavenging the harmful ROS may be a good strategy to prevent from ALD.

Microalgae are the generic term of unicellular algae, including flagellate, diatom, cyanobacteria, and green algae

[11]. Microalgae have been extensively utilized to produce biofuel and biodiesel while nowadays there has been a growing demand for isolating new bioactive peptides from the marine microalgae [12]. It has been reported that marine microalgae (*Chlorella*, *Dunaliella salina*, and *Arthrospira*) can be applied in cosmaceuticals, nutraceuticals, and functional foods, because there are rich in protein, lipid, vitamins, and other nutrients [13]. Some algae have been mass-cultivated as a nutritional supplement so far [14]. A few studies have indicated the bioactive peptides with antioxidant activity from marine microalgae such as *Tetradesmus obliquus* (a green microalga) and *Navicula incerta* (benthic diatom) [15]. Among the microalgae, there are few studies on flagellate. *Isochrysis zhanjiangensis*, distributed widely in the South China Sea, is applied to different fodder industry and mariculture systems, such as bivalves larvae culture [16]. As a marine golden-brown flagellated microalga, *I. zhanjiangenisis* can accumulate lipids contents under nitrogen-replete conditions, and has a potential to produce biodiesel using N-rich wastewater [17]. However, there are still no systematic investigations concerning biologically active compounds derived from *I. zhanjiangenisis*.

The way by means of enzymatic hydrolysis method of proteins has been used to produce peptides with different biological activities [18]. Biologically active peptides may be released by enzymatic breakdown involving the digestion of food proteins [19]. These peptides range in size from 2 to 20 amino acid residues [20], and have some biological properties, including antioxidant, antidiabetic, antihypertensive, immunomodulating, and growth-promoting functions [21].

It has been reported the bioactivities from enzymatic hydrolysis of *Navicula incerta*, but there are few studies on antihepatocyte peptides [22]. Based on those theories and facts, in this study, the hydrolysates of *I. zhanjiangensis* with various enzymes were performed to determine their free radical scavenging abilities. We also investigated hepatoprotective effects of the purified peptide from *I. zhanjiangensis* (PIZ) on ethanol-induced human hepatocellular carcinoma (HepG2) cells injury. This provides a new idea for *I. zhanjiangenisis* study.

2. Materials and Methods

2.1. Materials

HepG2 cells were purchased from American Type of Culture Collection (Manassas, VA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylltetrazolium bromide (MTT), chymotrypsin, trypsin, pepsin, dimethyl sulfoxide (DMSO), and 2',7'-dichlorofluoresin diacetate (DCFH-DA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modification of Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and trypsin were obtained from Gibco BRL, Life Technologies (Grand Island, NY, USA). Primary and secondary antibodies were provided from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). ECL kit was provided from GE Healthcare. Chemicals not mentioned were of analytical grade.

2.2. Cultivation of the microalga, *I. zhanjiangensis*

I. zhanjiangensis was cultured with f/2 culture medium [23] at 25 ± 1°C in sterilized natural seawater with 2% CO_2. The preparation of f/2 culture medium refers to this method [23]. Dry microalgae powder was obtained by concentration and lyophilized.

2.3. Enzymactic hydrolysis, *in vitro* gastrointestinal digestion, and amino acid compositions of *I. zhanjiangensis* enzymatic hydrolysate with *in vitro* gastrointestinal digestion (IZHG)

Chymotrypsin, trypsin, and pepsin with different specificities were required to degrade *I. zhanjiangensis*, respectively [24]. *I. zhanjiangensis* and enzyme (w/w, 100:1) were mixed, stirred, and incubated with optimal temperature in a reactor (10 L). 8 h later, it was heated (100°C, 20 min) aimed at inactivating the enzyme. After lyophilization, the hydrolysates were stored (-80°C) for further use.

The *in vitro* gastrointestinal digestion process was performed with the following method [25]. *I. zhanjiangensis* solution (4%, w/v) was adjusted to the optimal pH on behalf of the stomach digestion. Pepsin and *I. zhanjiangensis* (w/w, 1:100) were mixed and incubated (37°C, 2.5 h). Then pH was adjusted to 2.5. Trypsin and chymotrypsin were added to the previous mixture (enzyme/*I. zhanjiangensis*, w/w, 1:100, respectively). The solution was incubated (37°C, 2.5 h). The supernatant was obtained through concentration (4°C, 80.496 ×g, 20 min). After lyophilization, dry powder was stored at -80°C. Degree of hydrolysis (DH) was detected with trinitrobenzenesulfonic acid method [26], respectively.

Fifty milligrams of IZHG was dissolved with HCl (6 M, 2 mL) under vacuum (105°C, 24 h) and appropriately diluted. Amino acid was quantified as described by Kang *et al.* [27].

2.4. Electron spin resonance (ESR) measurement

Radical scavenging activity was calculated with following equation [28]:

$$\text{Radical scavenging activity} = \frac{1-H}{H_0} \times 100\%$$

H and H_0 represented relative peak height of radical

signals with and without sample, respectively.

2.4.1. 2,2-Diphenyl-1-picrylhydrazy (DPPH) radical scavenging assay

DPPH radical scavenging ability was evaluated utilizing the method as described by Gao *et al.* [29]. That is, the enzymatic hydrolysate or purified peptide (50 μL) was added to DPPH (100 μM, 50 μL) dissolved with ethanol and stirred (20 s). The mixture was injected into a quartz capillary (100 μL). Two minutes later, the spin adduct was determined with JES-FA ESR spectrometer. Measurement conditions: modulation frequency, 100 kHz; magnetic field, 335.00 ± 5 mT; microwave frequency, 9.43 GHz; microwave power, 5 mW; sweep time, 0.5 s; temperature, 25°C.

2.4.2. Hydroxyl radical scavenging activity assay

Fenton reaction was utilized to perform by reacting $FeSO_4$ (5 mM, 25 μL) and H_2O_2 (5 mM, 25 μL) to produce hydroxyl radicals [30]. The conditions were as follows: generated radicals were reacted with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) (0.5 M, 25 μL). And the sample solution (25 μL) or PBS (25 μL, pH 7.2) as a control was added. Three minutes later, the mixture was transferred to a capillary tube. DMPO-OH adduct was detected with an ESR spectrometer. Measurement conditions: modulation frequency, 100 kHz; magnetic field, 335.50 ± 5 mT; microwave frequency, 9.44 GHz; microwave power, 1 mW; sweep time, 0.5 min; temperature, 25°C.

2.4.3. Superoxide radical scavenging activity assay

Superoxide radicals were produced via UV irradiated riboflavin/EDTA system [31]. The mixture including riboflavin, EDTA, DMPO (0.6 mM, 10 mM, 0.2 M, respectively) and sample solution was irradiated with UV lamp (365 nm, 60 s). The mixture was transferred into a capillary tube and the spin adduct was determined by an ESR spectrometer. Measurement conditions: modulation frequency, 100 kHz; magnetic field, 335.50 ± 5 mT; microwave frequency, 9.41 GHz; microwave power, 5 mW; sweep time, 60 s; temperature, 25°C.

2.4.4. Alkyl radical scavenging activity assay

Alkyl radicals were produced by 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) [32]. The conditions were as follows: the reaction mixture including AAPH, 4-POBN (5mM, 5mM, dissolved in PBS (pH 7.2), respectively), and tested sample solution was incubated (37°C, 20 min) and then transferred to a 100 μL of capillary tube. The spin adduct was determined with an ESR spectrometer. Measurement conditions: modulation frequency, 100 kHz; magnetic field, 335.50 ± 5 mT; microwave frequency, 9.44 GHz; microwave power, 1 mW; sweep time, 240 s; temperature, 25°C.

2.5. Purification of antioxidant peptide

The gastro hydrolysate was purified by FPLC on a HiPrep 16/10 CM FF ion-exchange column equilibrated with sodium acetate buffer (20 mM, pH 4.0). The column was eluted with a linear gradient from 0 to 2.0 M NaCl at a flow rate of 1 mL/min. Each fraction was collected, detected at 280 nm, and lyophilized.

The fraction with higher free radical scavenging activity was further purified by RP-HPLC with a C_{18} column (20 mm × 250 mm) of Primesphere 10 with a linear gradient of acetonitrile from 0 to 30% in 0.1% trifluoroacetic acid (TFA) at a flow rate of 60 mL/h, and the eluent was monitored at 215 nm.

The active fraction was further purified by a Synchropak RPP-100 analytical column (4.6 mm × 250 mm) at a flow rate of 60 mL/h. Separation was performed through linear gradient elution (acetonitrile, 0-20%). The absorbance at 215 nm was detected as described above [24].

2.6. Amino acid sequence and molecular mass measurement

Sub-fraction from Synchropak RPP-100 column with relatively high free radical scavenging ability was analyzed through the electrospray ionization (ESI) source and Q-TOF mass spectrometer (MS) (Micromass, Altrincham, UK) [24]. The peptide was directly infused with methanol and water (v/v, 1:1) to the electrospray source. Molecular weight was evaluated via doubly charged $(M+2H)^{2+}$ state with the MS. After determining molecular weight, the peptide was automatically selected for fragmentation, and sequence information was obtained.

2.7. Cell culture and cytotoxicity assay *in vitro*

HepG2 cells were incubated (37°C, 5% CO_2) in DMEM (10% FBS and 1% penicillin/streptomycin). Cells were plated at 8×10^4 cells/well. After 24 h incubation, the cells were treated with PIZ (10, 20, 50, and 100 μM). Following 24 h incubation, 100 μL of MTT (1 mg/mL) solution was added and reacted (37°C, 4 h). 100 μL/well of DMSO was used to dissolve the formazan crystals. The absorbance of each well was detected at 570 nm with a microplate reader (BioTek, USA).

2.8. Cellular ROS analysis

Reactive oxygen species level was evaluated with DCFH-DA as the oxidation sensitive dye. HepG2 cells were grown with PIZ (10, 20, 50, and 100 μM). 1 h later, ethanol (0.75 M) was added and the cells were incubated for a day. After rinsing the cells thrice with DMEM, cells were

stained with DCFH-DA (10 μM) and incubated (37°C, 20 min). Fluorescence was measured by an invert fluorescent microscope (Olympus Opticals, Tokyo, Japan).

2.9. Western blot analysis

As described by Ji *et al.* [33], equivalent protein (10-30 μg) were separated electrophoretically utilizing SDS-PAGE (10%) and transferred onto a nitrocellulose (NC) membranes (Amersham, GE Healthcare Life Science, Germany). The membranes were blocked with 5% nonfat dry milk in TBST and incubated with primary and secondary antibodies including GGT (sc-100746), SOD (sc-271014), and Glutathione (sc-71155) mouse monoclonal antibodies and goat anti-mouse IgG-HRP (sc-2005) (1:500, 1:5000, respectively). Protein bands were evaluated with ECL Kit (GE Healthcare Life Science).

2.10. Molecular docking

Screening of 3D hGGT1 (ID: 4GG2) was provided from protein database. The three-dimensional structure of PIZ was drawn with Chemdraw 16.0 Molecular docking studies were performed for PIZ and GGT on Discovery Studio 3.5 (Accelrys, San Diego, CA, USA). CDOCKER protocol was utilized on evaluation of the molecular docking. DS 3.5 was used to describe the interaction between PIZ and GGT active sites [34].

2.11. Statistical analysis

All experiment data in triplicate are expressed as means ± standard deviation and significance was assessed as $p < 0.05$(*), $p < 0.01$(**), or $p < 0.001$(***). One way analysis of variance (ANOVA) was utilized. The differences between two groups were analyzed by Dunnett's Multiple Comparison Test. All analyses were performed using GraphPad Prism 5 for windows.

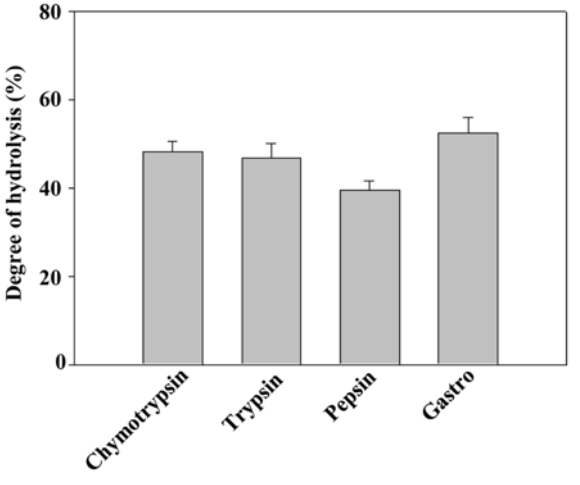

Fig. 1. Determination of degree of hydrolysis by trinitrobenzenesulfonic acid method. Values represent means ± SD (n = 3).

3. Results and Discussion

3.1. Preparation of *Isochrysis zhanjiangensis* hydrolysates and their free radical scavenging abilities

I. zhanjiangensis was hydrolyzed by chymotrypsin, trypsin, pepsin, and *vitro* gastrointestinal digestion. As shown in Fig. 1, the degree of hydrolysis for chymotrypsin, trypsin, pepsin, and gastro was 48.7, 46.9, 37.8, and 55.4%, respectively. The gastrointestinal hydrolysate exhibited strongest free radical scavenging abilities, especially hydroxyl radicals in Fig. 2.

Table 1 indicated amino acid compositions of the gastrointestinal hydrolysate. The composition was signified

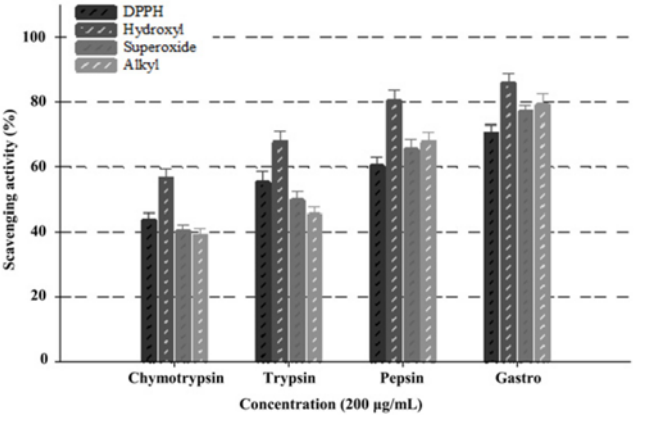

Fig. 2. Scavenging activities (DPPH, hydroxyl, superoxide, and alkyl) of hydrolysates by ESR spectrometer. Values represent means ± SD (n = 3).

Table 1. Amino acid compositions and contents of the *Isochrysis zhanjiangensis* hydrolysate with *vitro* gastrointestinal digestion

Amino acid	The hydrolysate with *vitro* gastrointestinal digestion (% AA/100 g)
Asp.	8.89
Thr.	3.12
Ser.	2.42
Glu.	3.59
Pro.	8.5
Gly.	23.34
Ala.	4.59
Val.	3.12
Ile.	3.9
Leu.	4.22
Tyr.	4.65
Phe.	9.3
His.	5.38
Trp.	3.01
Lys.	3.26
Arg.	6.25
Total	100

with amino acid residues/100 g. The hydrolysate was composed of amino acids, rich in 59.98% of hydrophobic Pro, Ala, Val, Gly, Ile, Leu, Phe, and Trp, 13.95% of aromatic Phe and Tyr, and 5.38% of His. There are some reports on the antioxidative efficacy of amino acids. For instance, tryptophan and histidine showed high antioxidant activity whereas glycine and alanine showed only weak activity, and methionine and cysteine had an antioxidative effect in Soybean oil [35]. And histidine-containing peptides possessed a higher activity [36]. This provides clues for seeking antioxidant substances existing in the enzymatic hydrolysates.

3.2. Purification of antioxidant peptide

FPLC method was used to separate the gastrointestinal hydrolysate to four fractions in Fig. 3A. And the free radicals scavenging of fraction 3 was higher than that of fractions in Table 2. The fraction 3 was further purified using RP-HPLC method on a Primesphere 10 C_{18} column, and three peaks were monitored at 215 nm in Fig. 3B. Fraction 3-3 showed strongest free radicals scavenging activities (Table 2). Fraction 3-3 continued to be purified via RP-HPLC with Synchropak RPP-100 column and divided into two fractions (Fig. 3C). Fraction collected was further identified for its free radical scavenging activity. And fraction 3-3-2 was used as its free radical scavenging activity was higher, compared with fraction 3-3-1. And fraction 3-3-2 was analysed as the following amino acid sequences: NDAEYGICGF (1088.16 Da) by Q-TQF ESI/MS (Fig. 4).

Generally, the molecular weight of antioxidant peptides is between 500-1800 Da [37]. And the presence of antioxidant peptides contributes to demic digestion and absorption [38]. Absorption is the process by which matter extracts other physical objects or energy. Demic digestion refers to that human digestive organs turn food into a process that can be absorbed by the body; the process of converting macromolecules such as starch, protein, and fat

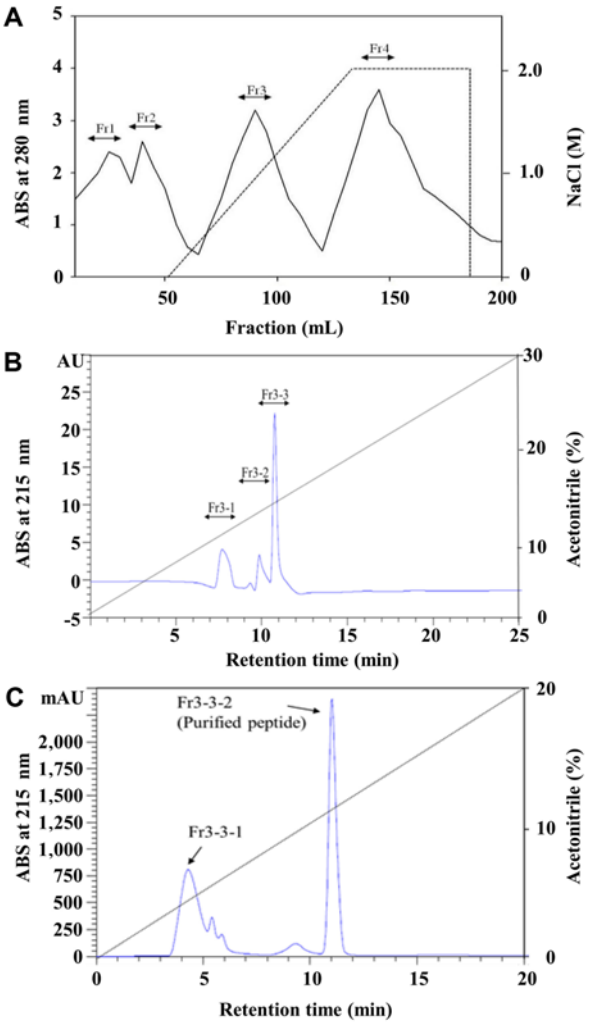

Fig. 3. (A) FPLC of the gastro hydrolysate by HiPrep 16/10 CM FF ion column. Adsorption peptides were eluted with a linear gradient of NaCl (0-2.0 M) at a flow rate of 60 mL/h. (B) RP-HPLC (Primeshpere 10 C_{18} column) for the purification of antioxidant peptides. Elution was performed with the linear gradient of acetonitrile (0-30%) containing 0.1% TFA at a flow rate of 1.0 mL/min. (C) RP-HPLC on a Synchropak RPP-100 analytical Column. Elution was performed with a linear gradient of acetonitrile (0-20%) containing 0.1% TFA at a flow rate of 1.0 mL/min.

Table 2. Four free radical scavenging abilities of each fraction

Fraction number (100 μg/mL)	Free radical scavenging activity (%)			
	DPPH	Hydroxyl	Superoxide	Alkyl
Fr1	40.2 ± 1.3	28.2 ± 2.3	6.2 ± 0.8	8.2 ± 2.3
Fr2	38.5 ± 1.7	48.2 ± 2.2	36.5 ± 1.2	44.2 ± 2.6
Fr3	75.8 ± 2.8	74.8 ± 3.2	64.1 ± 1.6	67.9 ± 1.5
Fr4	60.5 ± 1.8	70.5 ± 1.2	60.3 ± 2.3	60.5 ±2.7
Fr3-1	70.2 ± 2.0	72.7 ± 2.1	68.3 ± 2.5	70.5 ± 1.8
Fr3-2	71.1 ± 2.8	75.6 ± 2.6	65.8 ± 2.3	72.9 ± 2.1
Fr3-3	78.6 ± 2.7	76.4 ± 2.0	72.8 ± 2.4	71.2 ± 2.3
Fr3-3-1	74.6 ± 1.8	78.4 ± 2.5	74.2 ± 1.6	73.4 ± 2.2
Fr3-3-2	82.3 ± 2.7	86.2 ± 1.9	79.5 ± 2.3	75.2 ± 3.0

Values are mean ± SD of triplicates.

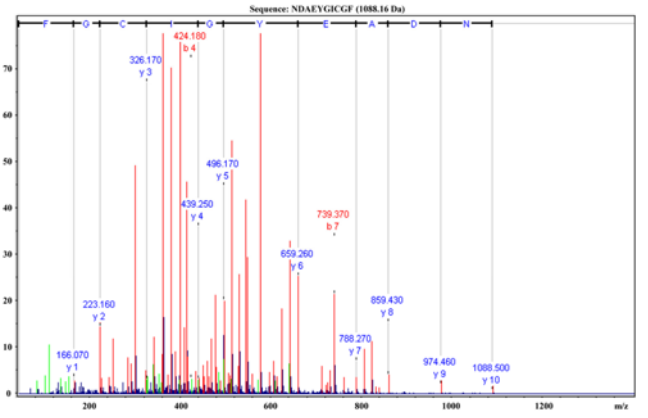

Fig. 4. Identification of molecular mass and amino acid sequence of PIZ. MS/MS experiments were performed on a Q-TOF tandem mass spectrometer (Micromass Co., Manchester, UK) equipped with a nano-electrospray ionization source. Sequencing of active peptide was acquired over the *m/z* range 50-2500 and sequenced by using the PepSeq de novo sequencing algorithm.

into foods into small molecules that are soluble in water [39-42]. The amino acid sequences and hydrophobicity also affect the antioxidant activity of the peptides. Almost all the active peptides have a Pro, His, Tyr, Trp, Met, and Cys in the structure [27]. PIZ structure may be one of the factors affecting its antioxidant activity [43]. Therefore, amino acid composition, sequence, and molecular size are of great importance for the antioxidant peptides.

3.3. Cell viability analysis

The toxicity of ethanol (0-2 M) exposure to HepG2 cells was assessed by measuring the cell viability with MTT analysis. The result manifested that 0.75 M ethanol approximately decreased to 50% of cell viability (Fig. 5A). PIZ had no cytotoxicity on HepG2 cells (Fig. 5B). The protective effects of PIZ were tested in the range of 10-100 μM against ethanol-induced cytotoxicity. Comparing with the control and sample groups, pretreatment with PIZ significantly increased cell viability (Fig. 5C).

3.4. Scavenging of intracellular ROS by PIZ

For the purpose of determining whether the prevention of ALD was relative to the suppression of ROS generation, a fluorescence probe DCFH-DA was employed. DCFH-DA was cleaved by intracellular esterases into DCFH (non-fluorescent). Then DCFH is oxidized by cellular ROS to produce DCF (fluorescent).

The fluorescence signal of DCFH-DA-stained cells (the ethanol-induced group) was higher, compared with the blank group (Fig. 6). Following PIZ pretreatment, this fluorescence signal decreased. And these results suggest

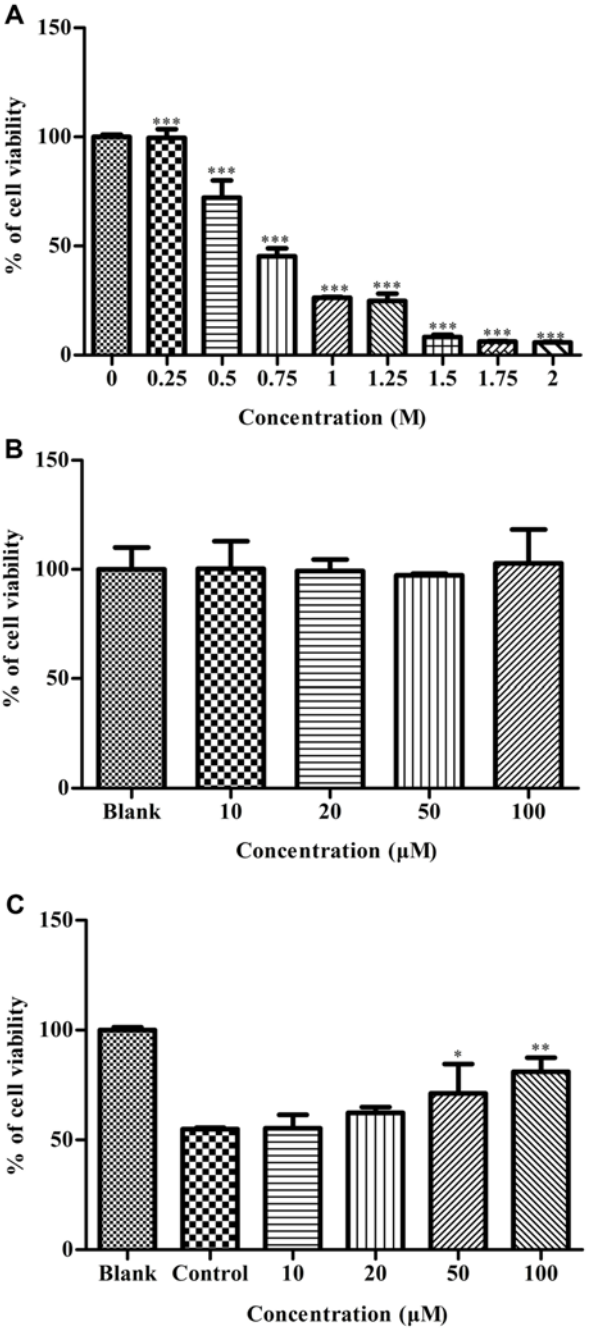

Fig. 5. (A) Cell viability in ethanol-injuried HepG2 cell. Cells were challenged with ethanol (0-2 M). (B) The toxicity of PIZ on HepG2 cells. Cells were cultured with PIZ for 24 h and measured by MTT assay. (C) Protective effect of PIZ on HepG2 cells induced by 0.75 M alcohol. Cells were pre-incubated with PIZ (10, 20, 50, and 100 μM). After the treatment, MTT analysis determined the cell viability. Data are shown as means ± SD (n = 3). Bars with different characters are statistically different at $p < 0.05$(*), $p < 0.01$(**) or $p < 0.001$(***). (A) and (B), compared with the blank group. (C), compared with the control group.

that PIZ pretreatment can effectively reduce ROS production to prevent from oxidative damage induced by alcohol.

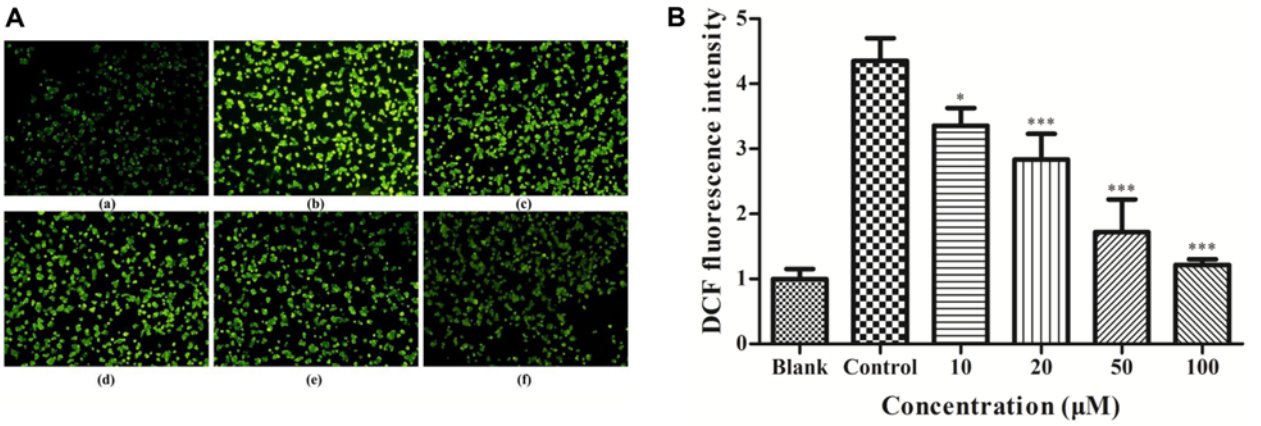

Fig. 6. Intracellular ROS was detected in HepG2 cells by using an invert fluorescent microscope. (A) (a) the blank group; (b) exposure to 0.75 M ethanol (the control group); (c) 10 μM PIZ; (d) 20 μM PIZ; (e) 50 μM PIZ; and (f) 100 μM PIZ for 24 h. (B) Values represent means ± SD (n = 3). Bars with different characters are statistically different at $p < 0.05$(*), $p < 0.01$(**) or $p < 0.001$(***) compared with the control group.

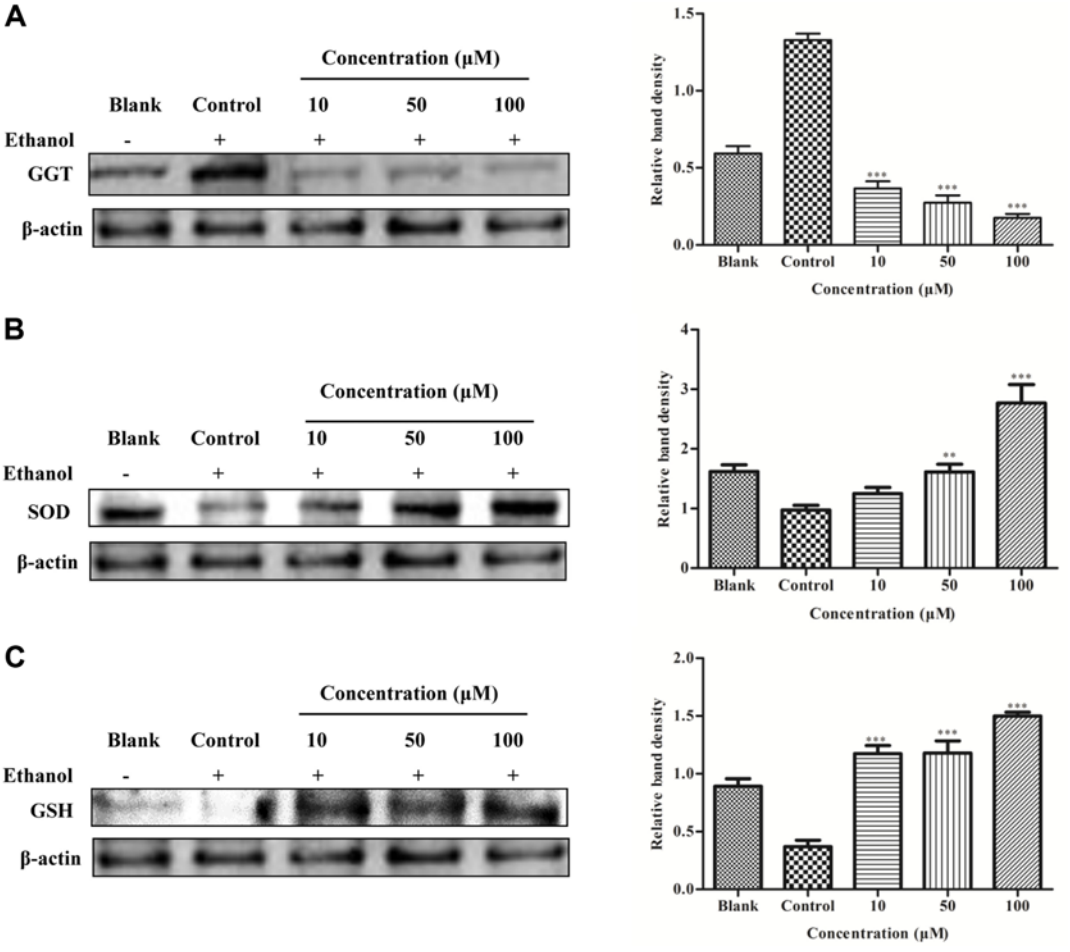

Fig. 7. Effects of PIZ on the release of GGT (A), SOD (B), and GSH (C). Data are shown as means ± SD (n = 3). Bars with different characters are statistically different at $p < 0.01$(**) or $p < 0.001$(***), compared with the control group.

3.5. Effect of the PIZ on SOD, GSH, and GGT protein expression

Mechanisms for ROS production in biological systems are represented by both non-enzymatic and enzymatic reactions [44]. SOD can catalyze the dismutation of two superoxide anions to hydrogen peroxide and oxygen [45]. Glutathione

(GSH) is among the most important antioxidants in cells, being used in enzymatic reactions to eliminate peroxides [46]. GGT, a marker of alcohol abuse and liver disease, is an enzyme involved in transport of amino acids and peptides in cells [47]. GGT is capable of instantly degrading endogenous GSH and exogenous GSH stimulated by external conditions, such as intake of ethanol, UV light, and toxins [38]. Furthermore, GGT level of the patients with alcohol intoxication is significantly elevated, beneficial to the diagnosis of ALD.

Levels of SOD, GSH and GGT were evaluated by western blot. Compared with the control group, as the concentration of the sample increased, the expression levels of SOD and GSH increased, while the expression of GGT decreased in Fig. 7. The data suggest that PIZ treatment may prevent ethanol-induced oxidative stress damage through the regulation of SOD, GSH, and GGT expression.

Previous studies showed that excessive of ROS may lead to severe damage to DNA, RNA, and proteins [48]. Thus, the effect of PIZ on levels of GGT, SOD, and GSH was likely due to its scavenging ROS ability. It is commonly believed that many anti-oxidative peptides include hydrophobic amino acid residues (Val or Leu) and aromatic amino acids (Phe and Tyr) [49]. And the bioactivity of a peptide is related to molecular weight [50]. PIZ, acting as antioxidant, is connected with its molecular weight, composition, amino acids sequences, structure, and hydrophobicity. It includes hydrophobic amino acids (Ala, Gly, and Ile), aromatic amino acids (Phe and Tyr), and Cys. These amino acids could display free radical quenching activity. PIZ possessing significant antioxidant activity could represent a relevant source for reducing hepatotoxicity.

3.6. Docking of GGT with PIZ

The molecular docking was demonstrated to find the binding site between PIZ and GGT and understand the interaction mechanism further. The CDOCKER results about PIZ-GGT combination can evaluate the rationality of side-chain backbone interactions, which is shown in Table 3. As can be seen the first way is best. Based on this result, a structural diagram of the docking of GGT and PIZ molecules was drawn (Fig. 8A). As we all know, hydrogen bonds play an indispensable role in the structure and function of biological molecules [51]. PIZ generated 8 hydrogen bonds with bond length of 4.1 Å, 4.4 Å, 3.5 Å, 4 Å, 6.2 Å, 3.7 Å, 4.1 Å, and 3.3 Å with Ser452, Thr381, Gly473, Lys562, Tyr403, Gly173, Asn401, and Arg327 (Fig. 8B). The molecular docking score and hydrogen bond interactions predicated that the PIZ has potential to interact with GGT active site.

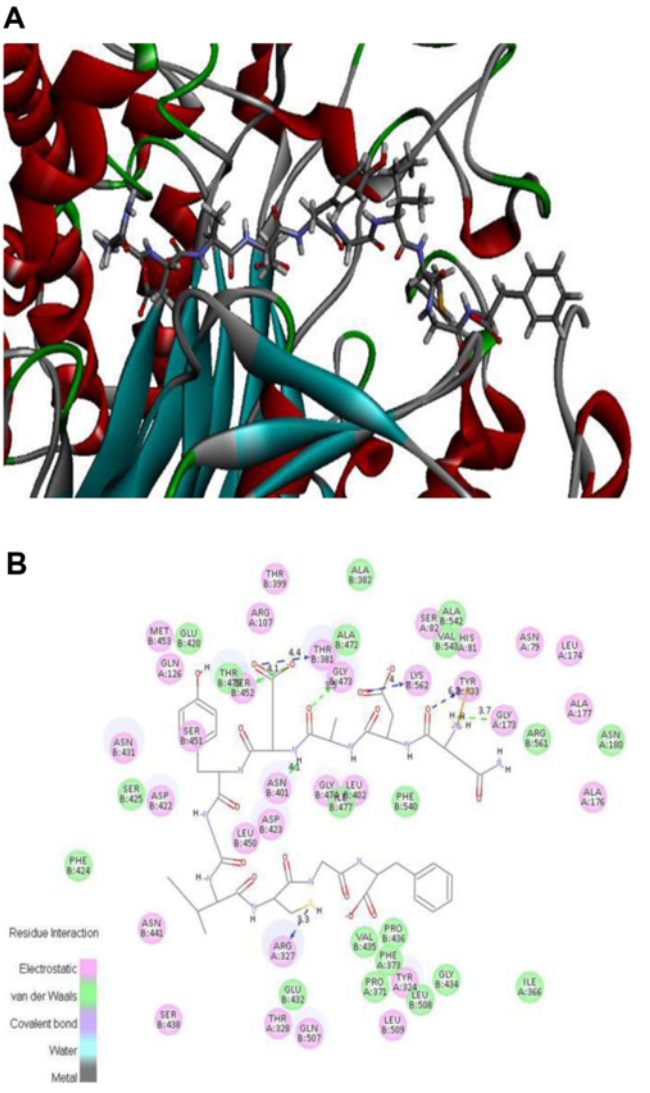

Fig. 8. The specific interactions between PIZ and GGT after the peptide automatic docked at GGT-active site. (A) Details of GGT-PIZ interactions. (B) The 2D diagram of interactions between PIZ and GGT.

Table 3. The Flexible Docking results of interactions between PIZ and GGT

Peptide	-CDOCKER_ENERGY (kJ/moL)	-CDOCKER_INTERACTION_ENERGY (kJ/moL)	POSE_NUMBER
PIZ	47.1	70.7	1
	-62.8	1.81	2
	-640.4	-98.3	3

4. Conclusion

In conclusion, PIZ was purified from gastro hydrolysates of microalgae, *I. zhanjiangensis*. And these results indicate that PIZ attenuates the ethanol-induced cytotoxicity of HepG2 cells and makes a difference in the prevention of ALD through inhibition of ROS production, which increase expressions of antioxidant enzymes (SOD and GSH). Consequently, it appears that PIZ has a potential effect as a food supplement on prevention of hepatotoxicity.

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