



Antioxidant and protective effects of a peptide (VTAL) derived from simulated gastrointestinal digestion of protein hydrolysates of *Magallana gigas* against acetaminophen-induced HepG2 cells

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Abstract

Oxidative stress is an automatic mechanism responsible for the commencement and continuance of liver injury. In this study, an antioxidative peptide Val-Thr-Ala-Leu (VTAL) was purified from simulated gastrointestinal digestion of protein hydrolysates of the triploid oyster *Magallana gigas*. Significant antioxidant activity was identified, as well as a protective effect against acetaminophen (APAP)-induced human liver cancer (HepG2) cells. The results suggested that the antioxidant activity improved in a dose-dependent manner. The highest cell viability ($88.105 \pm 3.62\%$) was observed in 15 mM APAP-induced cells when treated with 25 $\mu\text{g}/\text{mL}$ *M. gigas* peptide [M.g (pep)]. The peptide sequences include hydrophobic amino acids, which could be responsible for its chemoprotective and antioxidant activities. Treatment with M.g (pep) significantly promoted the proliferation of HepG2 cells, thus protecting them against APAP and imbuing them with significant antioxidant capacity. M.g (pep) could be beneficial for treating drug-induced oxidative stress and liver damage. Additionally, M.g (pep) could serve as an alternative to synthetic antioxidant drugs.

Keywords *Magallana gigas* · Enzymatic digestion · Bioactive peptide · Antioxidant activity · Chemoprotective effect · Acetaminophen · Simulated gastrointestinal digestion

Introduction

Oysters are highly nutritious marine organisms with medicinal value. They have a high protein, active polysaccharide, taurine, vitamin, and mineral content, and are also low in fat

(Guo et al. 2020). Bioactive materials have been extracted from various oyster species: oyster protein can be defragmented as multiple peptides with high bioactivity. Oyster protein hydrolysates (OPHs) and peptides are valuable owing to their stability and diverse biological activities (Guo et al. 2020; Xie et al. 2018). Many studies have described the bioactive properties of oyster peptides (OPs), including their antioxidant, antitumor, antimelanogenic, immunomodulatory, antifatigue, antimicrobial, antithrombotic, antiviral, antiwrinkle, antihypertensive, antiinflammatory, antifungal, anticancer, anticoagulant, and osteogenic effects (Ulagesan et al. 2022; Asha et al. 2016; Wang et al. 2014; Wang et al. 2010; Gueguen et al. 2006; Liu et al. 2008; Seo et al. 2013; Zeng et al. 2008; Hwang et al. 2012; Qian et al. 2020; Liu et al. 2007; Cheong et al. 2013; Han et al. 2019; Kim et al. 2015; Hao et al. 2013; Miao et al. 2018; Cheng et al. 2018; Cheng et al. 2021; Chen et al. 2019a, b A; Chen et al. 2019a, b B; Ngo et al. 2012; Bharathiraja et al. 2017). Oyster peptides may also enhance spatial learning and memory, acetylcholinesterase activity, and sexual function, and they also

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serve as angiotensin-converting enzyme (ACE) inhibitors (Wang et al. 2020; Zhang et al. 2021; Hao et al. 2022).

Acetaminophen (APAP) is a widely available antipyretic and painkiller medicine that has been used extensively since 1955. It is considered safe at therapeutic levels, but overdose or therapeutic misuse may cause hepatotoxicity and liver damage. In the last few decades, there have been concerns regarding the role of APAP in acute liver failure (ALF) in adults, with APAP-related ALF accounting for about 30–50% of cases worldwide (Bunchorntavakul and Reddy, 2013). In the USA, APAP hepatotoxicity was reported as the cause of 50% of all cases of overdose-related ALF and 20% of liver transplants. Toxic ingestion of APAP induces hepatic failure at concentrations above 150 mg/kg, but even minor doses may result in acute liver injury or failure (Yoon et al. 2016). During the COVID-19 epidemic, infected patients were widely treated with antipyretic agents that contained APAP (Feng et al. 2020). Misuse of these medicines could lead to liver injury (Zhang et al. 2022). Additionally, an APAP overdose will increase oxidative stress and levels of reactive oxygen species (ROS).

ROS are produced in the body by several endogenous systems (Lobo et al. 2010). The formation of free radicals, such as superoxide anions (O_2^-) and hydroxyl radicals ($\cdot OH$), are inevitable in aerobic organisms during respiration, and play a major role in many diseases. These radicals are highly unstable and so they react with other groups and elements in the body and cause cell or tissue injury (Kim et al. 2007; Bharathiraja et al. 2017). Excessive amounts of these free radicals induce oxidative stress, which may damage cells and lead to chronic diseases such as hepatitis, as well as alcoholic and nonalcoholic fatty liver diseases. Toxins, drugs, and xenobiotics are metabolized in the liver, while liver cells balance ROS production. Generally, the metabolic functions of the liver, and its relationship to the gastrointestinal tract, make it susceptible to these toxins (Cichoż-Lach and Michalak 2014; Bharathiraja et al. 2018; Santha Moorthy et al. 2017). Thus, antioxidant therapy with bioactive peptides could be useful to maintain the balance between oxidants and antioxidants during liver disease, as well as for protecting hepatocytes from oxidative stress.

Oysters are an abundant marine resource with a high protein content. Oyster proteins are digested by various proteases, producing low-molecular-weight protein hydrolysates and amino acids. Various enzymes are used to digest oyster proteins, and bioactive peptides have been purified from OPHs. Oyster peptides possess a wide range of bioactive properties that vary according to the receptors involved. Many studies have shown that OPs are derived from gastrointestinal enzymatic hydrolysates with high antioxidant activity (Qian et al. 2008, 2020; Nam et al. 2015). However, limited studies have been carried out to assess the

chemoprotective effects of OPs. This study aims to investigate the ability of OPs derived from *Magallana gigas* to repair the damage caused by APAP-induced hepatotoxicity using HepG2 cells.

Materials and methods

Chemicals

The following enzymes and chemicals were used in this study: trypsin, pepsin, α -chymotrypsin, fluorescein (FL), 2,2'-azobis(2-methylpropionamide) dihydrochloride (AAPH), (\pm)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), and APAP. All of these analytical grade reagents and enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reactions were performed in deionized water.

Sample collection and preparation

The broodstock of cultivated Pacific oysters *M. gigas* was procured from the Myeongdeung Fisheries in Geoje, Gyeongsangnam-do, Korea. The shell length (SL), shell height (SH), and shell width (SW) were measured using a Vernier caliper. The total wet weight, soft tissue weight (STW), and total shell weight were calculated using an electronic balance. The oysters were dissected and the adductor muscle, digestive gland, gills, labial palps, mantle edge, and gonads were isolated. Each specimen was frozen in liquid nitrogen and stored at $-80^\circ C$.

Preparation of *M. gigas* protein hydrolysate

The protein was extracted from the gill tissue of *M. gigas*. Gill tissue (1 g) was homogenized with deionized water at a ratio of 1:3 v/w. The homogenate was modified to the optimum temperature and pH for the protein hydrolysate preparation.

Protein hydrolysates were prepared with three different enzymes as single hydrolysates and in combination, with an enzyme/substratum ratio of 1/100 (w/w) using buffers under optimum conditions.

Four different protein hydrolysates were prepared as follows:

1. Pepsin (10 mM glycine buffer; pH 2 at $37^\circ C$ for 2 h at 100 rpm)
2. Trypsin (50 mM sodium phosphate buffer; pH 8 at $37^\circ C$ for 4 h at 100 rpm)
3. α -Chymotrypsin (50 mM sodium phosphate; pH 8 at $37^\circ C$ for 4 h at 100 rpm)

4. Pepsin + trypsin + α -chymotrypsin (digested under optimum pepsin conditions for 2 h; the pH was changed to 8, and trypsin and α -chymotrypsin were added and incubated for 4 h at 37 °C and 100 rpm)

After digestion, the enzymes were deactivated by heating for 10 min at 100 °C. The hydrolysates were centrifuged at 10,000 \times g (4 °C) for 10 min, and the supernatants were stored for further study.

Antioxidant activity

Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay of *M. gigas* protein hydrolysates was conducted using FL as the probe. The programmed ORAC assay was performed on a Synergy HTX multi-mode microplate reader (BioTek, Winooski, VT, USA) with fluorescence filters, at excitation and emission wavelengths of 485 nm and 535 nm, respectively; 96-well black-bottomed plates were used. The reaction was carried out at 37 °C, and was initiated by thermal decomposition of AAPH (Zulueta et al. 2009).

FL stock solution was prepared by dissolving of 44 mg of FL in 100 mL of phosphate-buffered saline (PBS; 75 mM, pH 7.0), and was stored in a dark refrigerated condition. Additionally, the working solution (78 nM) was freshly prepared by diluting 0.167 mL of the stock solution with 25 mL phosphate buffer. The AAPH radical (221 mM) was freshly made by making up 600 mg of AAPH to 10 mL with PBS. The reference standard was a 20 μ M Trolox solution prepared daily with PBS from a 1 mM stock standard solution, and kept in the freezer at –20 °C. Since the ORAC assay is very sensitive, samples should be diluted properly prior to the analysis to avoid interference. Each well was filled with 150 μ L of FL (78 nM) and 25 μ L of the sample, blank (PBS), or standard (Trolox, 20 μ M). Then 25 μ L of AAPH (221 mM) was added. Owing to the low conductivity of polypropylene plates, measurement deviations between wells can occur. To avoid this, the plates were warmed to 37 °C for 15 min before adding AAPH. Fluorescence was measured instantly after the addition of AAPH and measurements were then taken every 2 min up to 60 min. The measurements were performed in triplicate. The ORAC value, expressed as mM Trolox equivalent (mM TE/mg) was calculated by applying the following equation:

$$\text{ORAC } (\mu\text{M TE}) = C_{\text{Trolox}} \cdot \frac{(AUC_{\text{Sample}} - AUC_{\text{Blank}})}{k / (AUC_{\text{Trolox}} - AUC_{\text{Blank}})}$$

where C Trolox is the concentration (μ M) of Trolox (20 μ M), k is the sample dilution factor, and AUC is the area

under the fluorescence decay curve of the sample, blank, and Trolox.

Trolox equivalent antioxidant capacity (TEAC) assay

This study utilized the method described by Re et al. 1999 with minor modifications, to measure an individual sample's ability to inhibit the ABTS radical (ABTS +) when compared with a reference antioxidant standard (Trolox). ABTS + is produced by a chemical reaction with potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$). The radical was prepared by adding 25 mL of ABTS (7 mM) to 440 μ L of $\text{K}_2\text{S}_2\text{O}_8$ (140 mM) in the dark and allowing it to stand at room temperature for 12–16 h (the amount of time required for radical formation). The working solution was prepared by diluting a volume of the previous solution in PBS (pH 7.4) until the absorbance at $k = 734$ nm was 0.7 ± 0.02 . Further dark incubation was performed for 5 min, followed by diluted ABTS working solution (200 μ L + sample or PBS, or 10 μ L of Trolox) added, and the absorbance at 734 nm (Zulueta et al. 2009) was determined using the Synergy HTX multi-mode microplate reader. In the range of 0–400 μ M, a Trolox calibration curve was generated, and inhibition percentages for the samples were interpolated to determine the concentration in Trolox equivalents (mM TE/mg). Each measurement was performed in triplicate.

Molecular weight cutoff for separation

The *M. gigas* protein hydrolysate was separated by an ultrafiltration membrane (Vivaflow 200 Laboratory Cross Flow Cassette; Sartorius, Gottingen, Germany).

The protein hydrolysate was separated into four different molecular weight fractions:

- > 10 kDa
- 10–5 kDa
- 5–3 kDa
- < 3 kDa

Analyses of antioxidant activity were performed on the four different fractions. Compared with the other three molecular weight fractions, < 3 kDa exhibited greater antioxidant activity. To further purify the activity-rich fraction < 3 kDa, anion exchange chromatography was performed.

Fast protein liquid chromatography (FPLC)

After further purification, the > 3 kDa hydrolysate was run on a HiPrep 16/10 diethylaminoethyl ion-exchange column (DEAE-FF) with the FPLC system (AKTA Prime Plus; GE Healthcare, Piscataway, NJ, USA). The freeze-dried 3 kDa

protein hydrolysates (20 mg/mL) was dispersed in 20 mM Tris–HCl buffer (pH 9.5). Further, 5 mL of samples were loaded onto a HiPrep 16/10 DEAE FF ion-exchange column equilibrated in 20 mM Tris–HCl buffer (pH 9.5), followed by an elution into a linear gradient of NaCl (0–1 M) at a flow rate of 2 mL/min. For each fraction (5 mL), the peaks were monitored at 214 nm, and fractions corresponding to those peaks were collected and pooled. The freeze-dried pooled fractions were subsequently analyzed for antioxidants and hydroxyl radical-scavenging activity. The fraction with the pooled fractions that exhibited highest antioxidant and hydroxyl radical-scavenging activities was additionally purified with high-performance liquid chromatography (HPLC) to find a pure peptide.

Preparative reverse phase (RP)-HPLC

Fractions with higher antioxidant were further purified by RP-HPLC using a Luna 5 μm C18(2) 100 Å, 250 \times 10 mm LC column (Phenomenex., Torrance, CA, USA), attached to the Dionex Chromeleon 7.2 chromatography system (Thermo Fisher Scientific, Waltham, MA, USA.). Separation was performed with solution A (0.1% formic acid in deionized water) and solution B (0.1% formic acid in acetonitrile) eluted with a linear gradient of acetonitrile (0–5 min, 90% of solution A and 10% of solution B; 6–55 min, 20% of solution A and 80% of solution B; 56–60 min, 20% of solution A and 80% of solution B; 60–66 min, 90% of solution A and 10% of solution B) at a flow rate of 1 mL/min. Absorbance was monitored at 214 nm.

Determination of peptide sequences by liquid chromatography–tandem mass spectrometry (LC-MS/MS)

The amino acid sequence was confirmed by de novo sequencing of peptides derived from proteolytic substances or extracted through LC–MS/MS. A micro quadrupole time of flight (Q-TOF) III mass spectrometer (255,748; Bruker Daltonics, Hamburg, Germany) was used to perform the MS analysis. Filtered (Minisart syringe filter; Sartorius; pore size, 0.45 μm) samples (10 μL) were injected through a Poroshell 120 EC-C18 (2.1 \times 100 mm, 2.7 μm ; Agilent) LC column and separated using the UltiMate 3000 system (Thermo Fisher Scientific). The separation was attained at a flow rate of 0.2 mL/min by using a gradient elution program, starting with 98% solvent AA [$\text{H}_2\text{O}/\text{FA} = 100/0.1$ (v/v)] and 2% solvent B (acetonitrile/ $\text{FA} = 100/0.1$ (v/v)) for up to 2 min. This was gradually changed to 70% solvent A and 30% solvent B at 20 min. The gradient then changed rapidly to 5% solvent A and 95% solvent B from 20 to 21 min, which was then maintained up to 26 min. The program then rapidly

changed to 98% solvent A and 2% solvent B at 27 min, which was maintained up to 35 min.

Further samples were subjected to the mass spectrometer via electrospray ionization (ESI+) via the subsequent MS parameters: capillary voltage, 3500 V; nebulizer flow, 0.8 L/min; dry gas flow rate, 5.5 L/min; dry temperature, 180 °C; transfer, funnel 1RF at 400 Vpp and funnel 2RF at 400 Vpp; ISCID energy, 0 eV; hexapole radiofrequency setting, 250; quadruple, ion energy of 5.0 eV; low mass of 300 m/z; collision cell collision energy, 7 eV; collision radiofrequency, 600; transfer time, 80 μs ; and pre-pulse storage, 10 μs . The collected spectra were scanned from 50 to 2000 m/z. The normalized collision energy of 100 Vpp was used to produce the MS spectra by the collision-induced dissociation of the metabolite ions. Peptide de novo sequencing was performed to analyze the amino acid sequence based on the MS/MS spectra. A single peptide amino acid sequence was identified.

Cell culture

HepG2 human liver cancer cells (cat. no. HB-8065) were procured from the American Type Culture Collection (Manassas, VA, USA) with the culture conditions of 37 °C with 5% CO_2 in minimum essential medium (MEM; Sigma-Aldrich) enhanced with 10% fetal bovine serum (FBS) (GenDEPOT, Katy, TX, USA) containing 100 U/mL penicillin and 100 mg/mL streptomycin. The medium was changed on alternative days.

Cell viability assay

Cell viability was assessed with the EZ-Cytox cell viability assay kit (cat. no. EZ-1000). Cells were seeded in 96-well plates (2×10^4 cells/well in 100 μL medium) and allowed to attach for 24 h at 37 °C. The attached cells were then treated with *M. gigas* (pep) (1.25, 2.5, 5, or 25 $\mu\text{g}/\text{mL}$) and 15 mM APAP (A7085; Sigma-Aldrich) in serum-free MEM (SFM) for 18 h at 37 °C. After adding the Cytox solution to the cells, they were incubated at 37 °C for 1 h, and the absorbance was measured at a wavelength of 450 nm using a FilterMAX F5 microplate reader (Molecular Devices LLC, San Jose, CA, USA) (Kim et al. 2019).

Apoptosis assay

Apoptosis was evaluated using Muse annexin V and a dead cell assay kit (cat. no. MCH100105; BD Biosciences, Franklin Lakes, NJ, USA). The harvested cells were washed twice with PBS, and stained with FITC annexin V and propidium

iodide for 15 min at room temperature. The apoptotic cell percentage was determined using annexin V and the Muse cell analyzer system (Merck Millipore, Burlington, MA, USA).

Statistical analysis

All the results are presented as the mean with the standard deviation of three independent experiments. The significant difference among the means was calculated by one- or two-way ANOVA followed by Dunnett's multiple comparison test using GraphPad Prism software (version 9/0; GraphPad Software Inc., San Diego, CA, USA). A p -value < 0.05 was considered to indicate a statistically significant difference.

Results

Protein extraction and purification

M. gigas (triploid oyster) was dissected and crude protein was extracted from the gill tissue.

Antioxidant activity of *M. gigas* protein hydrolysate

The oyster protein was digested with multiple proteases, either singly or in combination. The digested protein hydrolysates were analyzed to determine their antioxidant activities (ORAC and TEAC). The three enzymes

(pepsin + trypsin + α -chymotrypsin) combined in the simulated gastrointestinal digestion (SGID) had higher antioxidant activity than the other protein hydrolysates and unhydrolyzed *M. gigas* protein (Fig. 1a and b). The ORAC and TEAC values in the SGID were 3437.22 ± 44.7 and 392.96 ± 9.3 mM TE/mg, respectively, and the corresponding values for unhydrolyzed *M. gigas* were 311.371 ± 27.3 and 117.28 ± 9.3 mM TE/mg.

Antioxidant activities of different molecular weight fractions

The gastrointestinal digested protein hydrolysate was further purified using different molecular weight cutoffs (> 10 , 10 – 5 , 5 – 3 , and < 3 kDa). The lowest molecular weight fraction (< 3 kDa) had the highest antioxidant activity. The ORAC and TEAC values were $4,298.51 \pm 82.69$ and $5,44.91 \pm 7.73$ mM TE/mg, respectively (Fig. 2a and b).

Purification and antioxidant activity of < 3 kDa molecular weight fraction

Anion exchange chromatography (FPLC) was used to purify the antioxidant-rich (3 kDa) molecular weight fraction (Fig. 3a). Gradient elution was performed using 1 M NaCl. Six peaks were observed and pooled, and then analyzed for antioxidant activity. Fraction 3 exhibited the maximum antioxidant activity compared with other fractions (Fig. 3b and c).

Fig. 1 Antioxidant activity of *M. gigas* protein and the different protein hydrolysates of *M. gigas*. **a** ORAC activity, **b** TEAC activity. Values are expressed as mean \pm SD ($n=3$). *denote significant differences ($p < 0.05$), while ns indicate no significant difference ($p > 0.05$)

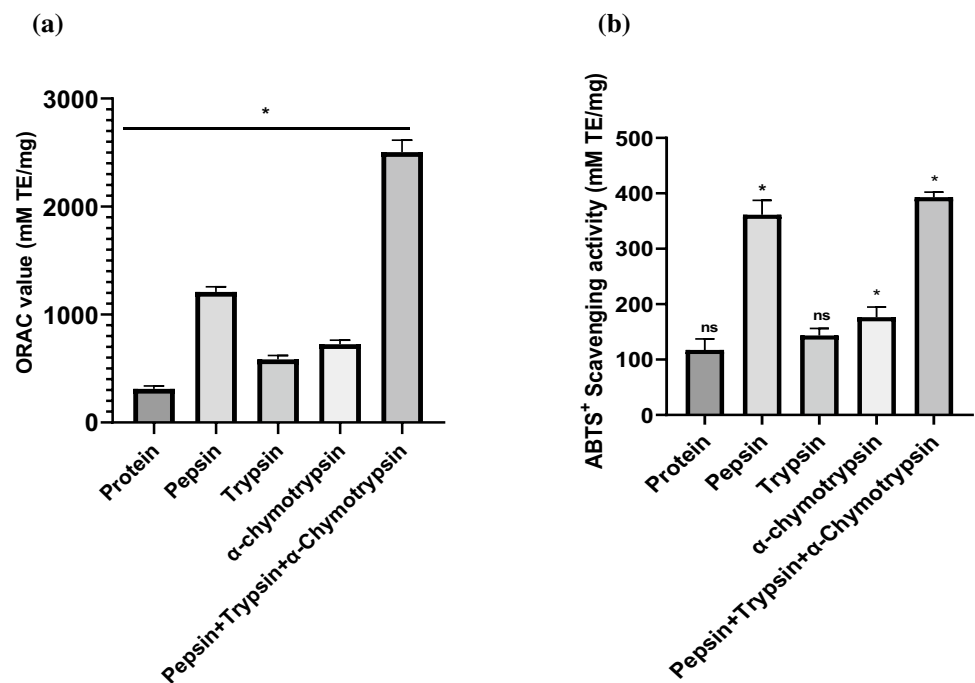


Fig. 2 The antioxidant activity of different molecular weight fractions of *M. gigas* protein hydrolysates. **a** ORAC activity, **b** TEAC activity. Values are expressed as mean \pm SD ($n=3$). * denote significant differences ($p < 0.05$), while ns indicate no significant difference ($p > 0.05$)

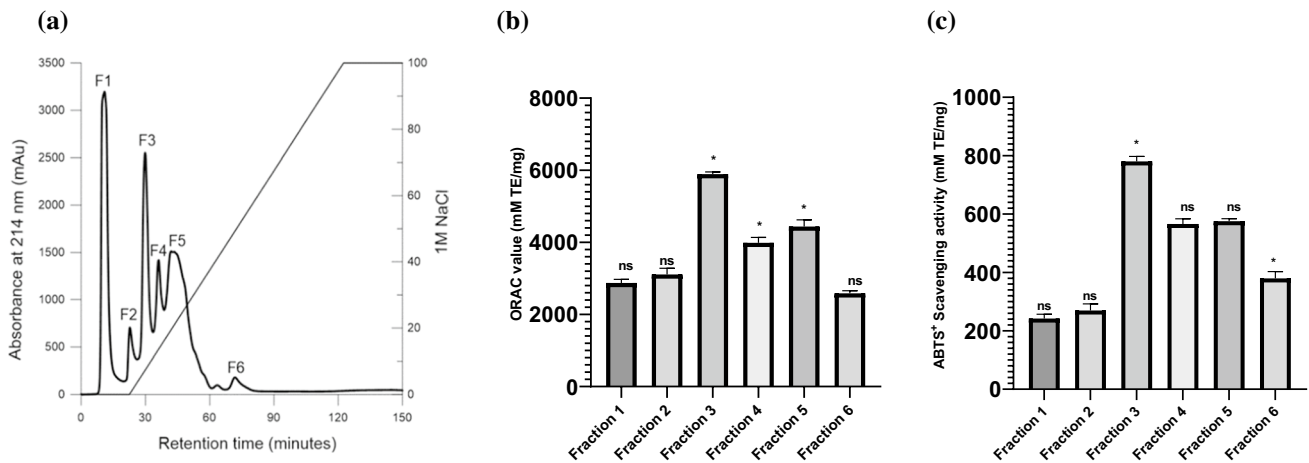
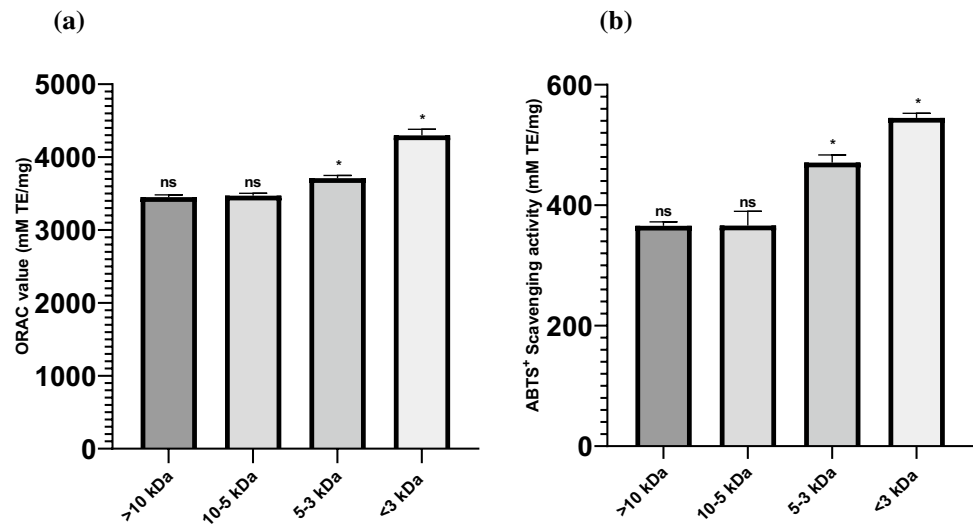


Fig. 3 **a** Fast protein liquid chromatography profile of <3 kDa peptide. A 5 mL sample was loaded onto a HiPrep 16/10 DEAE FF anion-exchange column. Separation was performed at 2 mL/min. Elution was monitored at 214 nm. Antioxidant activity of the fractions

collected from FPLC. **b** The ORAC value of the fractions, **c** the ABTS activity of the fractions. Values are expressed as mean \pm SD ($n=3$). *denote significant differences ($p < 0.05$), while ns indicate no significant difference ($p > 0.05$)

Purification of the antioxidant-rich fraction 3

Antioxidant-rich fraction 3 was further purified by RP-HPLC, with two peaks analyzed separately for antioxidant activity (Fig. 4a) and compared with glutathione (GSH) (naturally occurring antioxidant). Peak two had the highest antioxidant activity (Fig. 4b and c). Peak two (p2) had the maximum antioxidant activity and was subsequently analyzed by LC-MS/MS; a single peak was observed (Fig. 5) and the peptide sequence was identified by de nova sequencing (Table 1).

Analysis of *M. gigas* peptide [M.g (pep)] in APAP-induced HepG2 cell

Cell viability

The cell viability of M.g (pep) was analyzed relative to the APAP-induced toxicity in HepG2 liver cells. The APAP-treated groups had a 60% survival rate after an 18 h treatment. The cell viability of the APAP-treated group was $61.67 \pm 2.32\%$ that of the control (Fig. 6). Subsequent treatment with 15 Mm APAP and 1.25, 2.5, 5, and 25 $\mu\text{g}/\text{mL}$ M.g (pep) was associated with significantly higher cell viability compared with the control group: $69.20 \pm 3.66\%$, $79.78 \pm 7.3\%$, $85.97 \pm 2.5\%$, $88.10 \pm 3.6\%$, respectively.

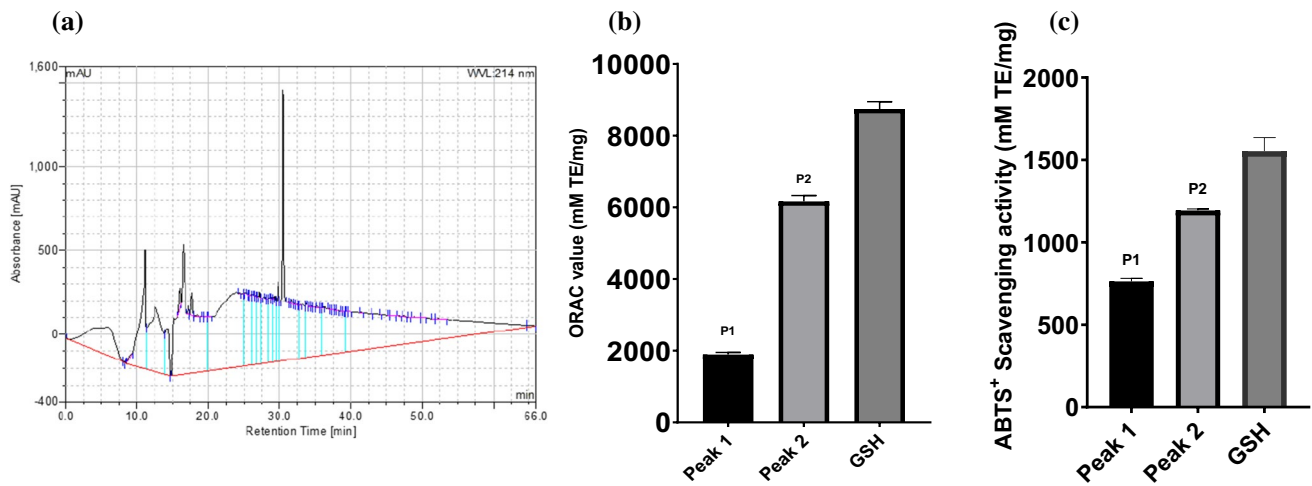


Fig. 4 **a** Results of RP-HPLC analysis using a Luna 5 μm C18 (2) 100 \AA , 250 \times 10 mm LC column attached to a Dionex system. Elution was performed with solution A (0.1% formic acid in deionized water) and solution B (0.1% formic acid in acetonitrile), eluted with a linear gradient of acetonitrile (0–80% in 0–66 min) at a flow rate of 1 mL/

min. Absorbance was monitored at 214 nm. Antioxidant activity of peaks identified by RP-HPLC. **b** The ORAC value of the fractions, **c** the ABTS activity of the fractions are compared with the glutathione (GSH)

These results suggested that the increased cell viability was evident in a dose-dependent manner.

Discussion

Oyster proteins generally contain various bioactive sequences, which can be released by enzyme hydrolyses to act as bioactive peptides. Thus, enzyme hydrolyses increase the nutritional properties of the oyster protein (Wang et al. 2010). The present study also elucidated the protective effects of a bioactive peptide derived from protein hydrolysates of the oyster *Magallana gigas* against acetaminophen-induced HepG2 cells. Oyster meat accounts for about half of the dry weight of an oyster (Linehan et al. 1999). The oyster gill is a complex ciliated organ with major roles in feeding, respiration, and excretion. Cilia of the gill are involved in the generation of strong water currents that pass through numerous branchial chambers to ensure gas exchange between organs and the surrounding medium, as well as the transport of food particles. Crude protein was isolated from the gill tissue of *M. gigas*. Protein hydrolysates digested with the combined SGID showed higher antioxidant activity. The antioxidant activity of gastrointestinally digested protein hydrolysates were significantly higher than other enzyme-digested protein hydrolysates and unhydrolyzed *M. gigas* protein. The antioxidant activities of protein hydrolysates mainly depend on the amino acid composition and sequence, as well as the peptide's configuration and size (Chen et al. 1996). Generally, antioxidant activity is characterized by the enzymes when using the same protein

substrates. Protein hydrolysates derived from different proteases had different antioxidant activities, suggesting that the enzymes were a major factor influencing the antioxidant properties of the protein hydrolysates. The results indicated that *M. gigas* undergoing SGID released more amino acids than other enzyme-digested protein hydrolysates. A previous study on SGID of mulberry leaf protein and its neurase hydrolysates found similar results (Sun et al. 2021). Further, the antioxidant activity of different molecular weight fractions indicated the lower fraction (< 3 kDa) exhibited the maximum activity, demonstrated with ORAC and TEAC assays. The molecular size of a peptide is also a major factor influencing the absorption of food protein hydrolysates. Peptides with lower molecular weights were the main contributors to the antioxidant activity of protein hydrolysates (Garcia-Mora et al. 2015). These results are in accordance with those of previous studies reporting that low-molecular-weight peptides have greater antioxidant activity (Ren et al. 2008; Zhu et al. 2008; Cao et al. 2009). The FPLC purified > 3 kDa fraction with higher antioxidant activity (fraction 3) was subjected to RP-HPLC analysis and further separated into two peaks. The peak with higher activity (P2) was further purified by LC-MS/MS and the peptide sequence was identified as Val-Thr-Ala-Leu (VTAL). A strong correlation exists between the amino acid composition and the bioactivities of peptides (Mendis et al. 2005a, b A; Mendis et al. 2005a, b; Shen et al. 2010). Generally, the higher the content of hydrophobic amino acids (Pro, Tyr, Val, Ala, Leu, Ile, Phe, and Met) in the peptide sequence, the stronger the antioxidant activity; this may be due to the interaction with lipid-soluble free radicals and a delay in lipid peroxidation

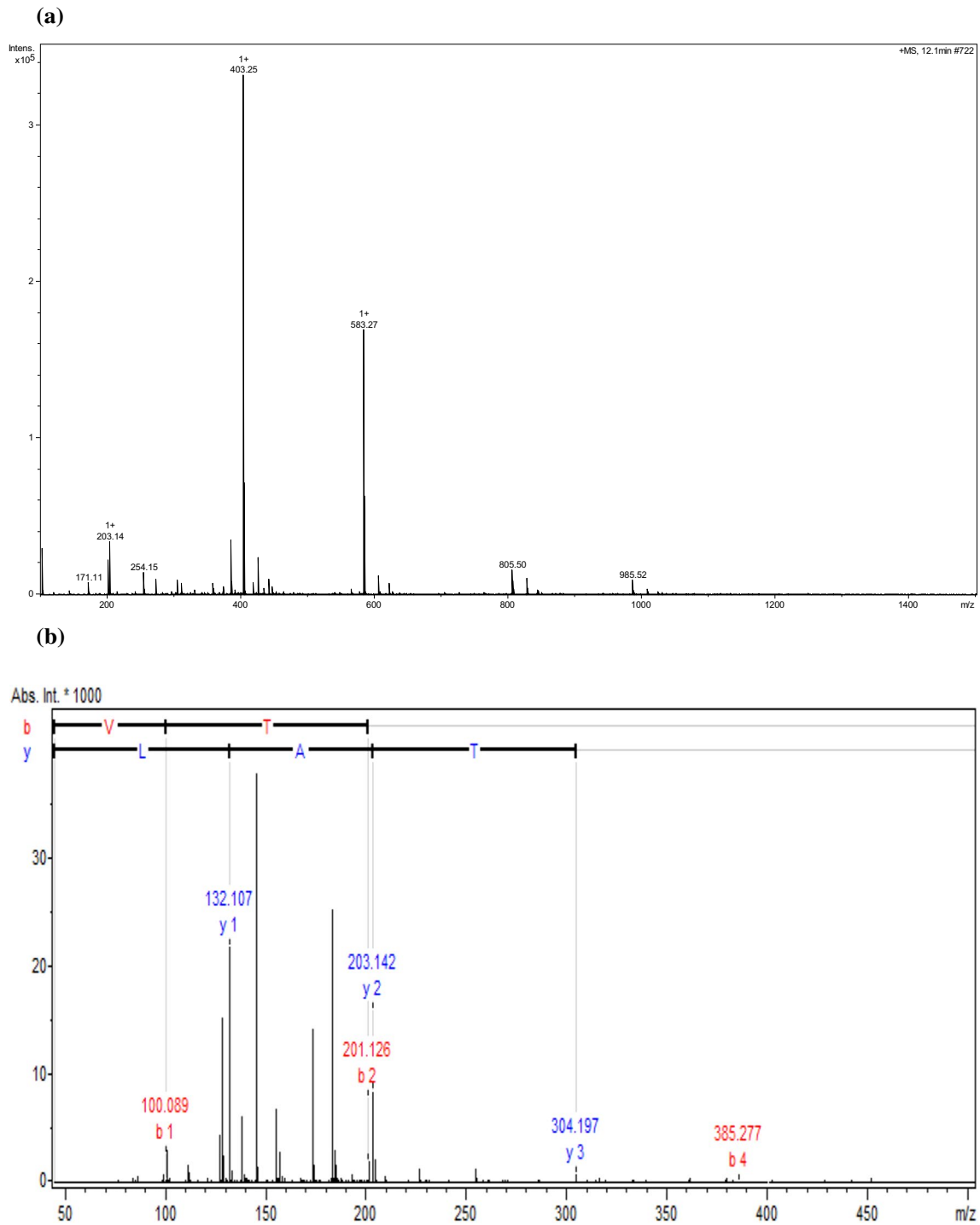


Fig. 5 The LC-MS/MS analysis of Peak 2. **a** MS spectrum of the peptide, **b** MS/MS sequencing

Table 1 The isolated peptide sequence

Charge	m/z	Sequence
1	403.25	VTAL

(Harnedy and FitzGerald, 2012; Bunchorntavakul and Reddy, 2013; Zou et al. 2016). The peptide sequence identified in the present study contained Val-Thr-Ala-Leu, which indicated that the peptide isolated from *M. gigas* was rich in amino acids implicated in antioxidant activity, and could therefore be a potential antioxidative peptide. Further, the

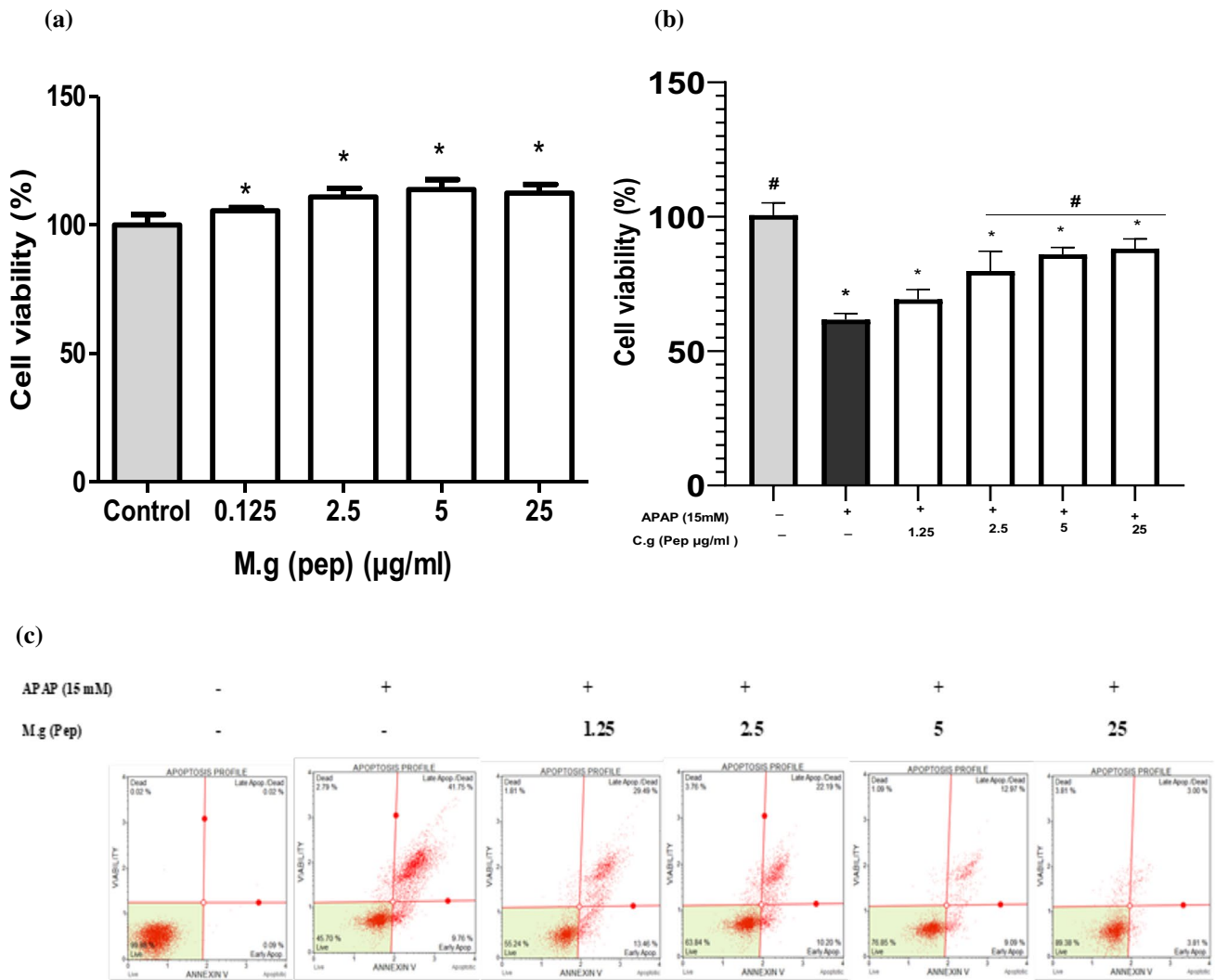


Fig. 6 Cell viability was measured by Cyttox assays, and the results are presented as the percentage of surviving cells compared with the control (non-treated group). Protective effects of M.g (pep) against APAP-induced liver damage involving HepG2 cells. **a** Cell viability of M.g (pep); **b** Protective effect of M.g (pep) against APAP c HepG2 cells were incubated with 15 mM APAP with or without various con-

centrations of PYP1– 4 for 18 h. FITC Annexin V flow cytometry was employed to determine the percentages of apoptotic and necrotic cells. Data are presented as the mean ± SD of three independent experiments and were analyzed using two-way ANOVA. *Indicate the significance value $p < 0.05$ versus control group, #denotes the significance value $p < 0.05$ versus the 15 mM APAP group

protective effect of protein hydrolysate was tested with APAP-induced HepG2 cells and increased viability was observed in a dose-dependent manner. Park et al. 2014 also revealed the protective effects of enzymatic oyster hydrolysate against APAP-induced HepG-2 cell damage, and the results show that the oyster hydrolysate has potential as a health food or liver-protecting drug.

This study showed that *M. gigas* protein hydrolysate subjected to SGID had significantly more antioxidant activity than other enzyme-digested protein hydrolysates and unhydrolyzed proteins. Furthermore, the low-molecular-weight peptide fractions derived using molecular weight cutoffs exhibited significant antioxidant activity. However,

the purified peptide (VTAL) also had remarkable antioxidant activity, among other positive effects (i.e., promotion of cell viability and reduction of oxidative stress) against APAP-induced liver damage involving HepG2 liver cells. The identified peptide sequence, which encompasses hydrophobic and aromatic amino acids, could promote antioxidant and chemoprotective activity. Further synthesis and in vivo studies of the peptide will be valuable for the food and pharmaceutical industries.

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by at least two professional editors, both native speakers of English. For a certificate, please see: <http://www.textcheck.com/certificate/3vC5LX>

Author contributions Conceptualization, SU, TJN, and YHC; formal analysis, SU and SJP; investigation, SU and TJN; data curation, SU and SJP; writing—original draft preparation, SU; writing—review and editing, YHC and TJN; supervision, YHC and TJN; project administration, YHC; funding acquisition, YHC. All authors have read and agreed to the published version of the manuscript.

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Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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