Original Article

Isolation of the hemeoxygenase-1 inducer from rice-derived peptide

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(Received 7 September, 2021; Accepted 17 November, 2021; Released online in J-STAGE as advance publication 25 January, 2022)

Bioactive peptides with various health benefits have been reported from rice protein hydrolysates. We previously showed that rice-derived peptides (RP) increased intracellular glutathione levels and induced the expression of γ-glutamylcysteine synthetase, which is regulated by nuclear transcription factor-erythroid 2-related factor 2 (Nrf2). Heme oxygenase-1 (HO-1) is an important Nrf2 downstream antioxidant enzyme that protects against oxidative stress. This study aimed to investigate the protective effects of RP on hydrogen peroxide (H_2O_2)-induced oxidative stress in human hepatoblastoma cell line HepG2 and identified HO-1 induced peptides from RP. Pretreatment of cells with RP reduced the cytotoxicity caused by H_2O_2 in a dose-dependent manner. Moreover, RP induced HO-1 expression in a concentration- and time-dependent manner. Next, we attempted to isolate the HO-1 inducer from RP by bioactivity-guided fractionation. Purification of the active peptides using a Sep-Pak C18 cartridge and reversed-phase HPLC, followed by sequence analysis by mass spectrometry, led to the identification of the three peptides. These peptides effectively reduced H_2O_2-induced oxidative stress. Among them, only P3 (peptide sequence: RSAVLLSH) increased HO-1 protein expression. Additionally, the knockdown of Nrf2 suppressed the induction of HO-1 expression by P3. Our results indicated that P3 identified from RP induced HO-1 by activating the Nrf2 signaling pathway.

***Key Words*: rice-derived peptides, cytoprotection, heme oxygenase-1, nuclear transcription factor-erythroid 2-related factor 2, reverse-phase high-performance liquid chromatography**

Reactive oxygen species (ROS) such as superoxide anion radicals, hydrogen peroxide, singlet oxygen, and hydroxyl radicals are generated as by-products of cellular metabolism. Overproduction of ROS can damage biomolecules such as DNA, lipids, and proteins, inducing cellular dysfunction and oxidative stress.[1] Oxidative stress has been implicated in many human diseases, including atherosclerosis, pulmonary fibrosis, neurodegenerative disorders, and cancer.[2,3] ROS formation is prevented by endogenous enzymatic and non-enzymatic antioxidants.[4] For example, polyphenols have been shown to act as antioxidants not only by quenching ROS directly, but also by activating the endogenous antioxidant defense system.[5]

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a key factor in the cellular antioxidant defense system, which regulates the expression of several phase II detoxifying/antioxidant enzymes.[6,7] Biliverdin can be converted into bilirubin by biliverdin reductase.[8] The end-products, CO and bilirubin are known for their antioxidant, anti-inflammatory, anti-apoptotic effects.[9,10] A previous study indicated that murine cells lacking HO-1 are susceptible to the accumulation of free radicals and to oxidative injury *in vitro* and *in vivo*, thus establishing that HO-1 is an important enzymatic antioxidant system.[11] Additionally, the induction of HO-1 expression contributes to protection against liver damage induced by acetaminophen (APAP).[12] HO-1 pathway is a key cytoprotective mechanism against oxidative stress and also plays a role in the maintenance of cellular homeostasis.[13]

Rice (*Oryza sativa* L.) is widely consumed worldwide, particularly in Asian countries. Rice-derived peptides have been reported to have various biological effects such as immune-modulating activity,[14] anti-oxidative activity,[15–17] anti-microbial activity,[18] antidiabetic activity,[19,20] and antihyperglycemic activity.[21] In our previous studies,[22,23] we demonstrated that rice-derived peptides (RP and Oryza Peptide-P60) increased intracellular glutathione levels in the human hepatoblastoma cell line HepG2 and induce the expression of γ-glutamylcysteine synthetase (γ-GCS), which is regulated by Nrf2. In addition, fermented brown rice extract was shown to elevate HO-1 mRNA levels in glioma cells.[24] However, there have been no reports evaluating the active components of RP with the induction of HO-1 expression.

In this study, we investigated whether RP induced HO-1 protein expression. Furthermore, we isolated the HO-1 inducer from RP using a Sep-Pak C18 cartridge and reversed-phase HPLC, and evaluated the function of these peptides using chemically synthesized peptides.

Materials and Methods

Materials. Denazyme AP, a protease from *Aspergillus oryzae*, was supplied from NAGASE Chemtex Co. Ltd. (Osaka, Japan). The commercial rice protein, Oryza Protein-P70, was a gift from Oryza Oil and Fat Chemical Co. Ltd. (Aichi, Japan). Minimum Essential Medium (MEM) was purchased from Sigma-Aldrich (St. Louis, MO). The Cytotoxic Detection Kit was purchased from Roche Diagnostics (Mannheim, Germany). The protein assay kit was purchased from Bio-Rad Laboratories Inc. (Hercules, CA). RIPA buffer was obtained from Santa Cruz Biotechnology (Dallas, TX). The polyvinylidene fluoride (PVDF) membrane was purchased from Bio-Rad (Hercules, CA). The PVDF Blocking Reagent for Can Get Signal® was purchased from Toyobo (Osaka, Japan). Anti-Nrf2 antibody was purchased from Abcam (Cambridge, United Kingdom). Anti-HO-1 antibody was purchased from Enzo Life Sciences (Farmingdale, NY). Anti-β-actin antibody was purchased from Sigma-Aldrich. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies and ECL reaction solution were obtained

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doi: 10.3164/jcbn.21-125

from GE Healthcare (Buckinghamshire, UK). Synthetic peptides were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA).

Preparation of RP. RP was prepared as previously described.[20] Briefly, 2 g of rice protein was suspended in 40 ml of distilled water, adjusted to pH 7.5 with 5 M NaOH, and incubated with 1% (w/w) Denazyme AP at 50°C for 17 h. The hydrolysates were heated at 80°C for 30 min, and centrifuged at 2,000 × *g* for 20 min. The supernatants were freeze-dried and stored at 4°C for further studies.

Cell culture. The human hepatoblastoma cell line HepG2 was purchased from the RIKEN BRC Cell Bank (Ibaraki, Japan). The cells were maintained in MEM supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES (pH 7.4), 0.56 μg/ml amphotericin B, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

Cytotoxicity assay. HepG2 cells were seeded at a density of 2×10^4 cells/well in a 96-well plate. The cells were treated with 2.5, 5, and 10 mg/ml of RP for 24 h. The medium was aspirated and replaced with FBS-free medium containing either 150 or 300 μM H_2O_2. After 24 h of incubation, the cell viability was determined. The culture medium was collected and used to measure LDH activity. LDH activity was measured using a Cytotoxic Detection Kit, as described in the manufacturer's instructions.

Western blotting analysis. HepG2 cells were seeded at a density of 3×10^5 cells/well in a 6-well plate. The cells were treated with 5 mg/ml RP for the indicated time periods. To evaluate the ability to induce HO-1 expression, cells were treated with each fraction for 24 h. After incubation, the cells were lysed using RIPA buffer for 30 min at 4°C. Lysates were centrifuged at 13,000 × *g* for 10 min at 4°C. Protein concentration was determined using a Bio-Rad protein assay. For immunoblot analysis, the prepared proteins were separated by SDS-PAGE and transferred electrophoretically to a PVDF membrane. The membrane was blocked with PVDF Blocking Reagent for 1 h at room temperature (23–25°C). Immunoblotting was performed using primary antibodies against HO-1, Nrf2, and β-actin overnight at 4°C. Bound antibodies were detected using secondary peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies. Target proteins were visualized using an ECL reaction solution and detected using an Amersham Imager 600 (GE Healthcare). Band intensities were quantified using ImageJ software (ver. 1.52a, National Institutes of Health, Bethesda, MD).

Isolation of HO-1 inducer from RP. RP was examined by column chromatography on a Sep-Pak C18 cartridge (Waters, Milford, MA) and was developed with 50 ml of 0, 2.5, 5%, 10%, 20%, and 40% acetonitrile (CH_3CN). Each eluate was freeze-dried and assayed in terms of the induced activity against HO-1. Based on the induction of HO-1 expression in HepG2 cells, the active eluate was further separated by reverse-phase HPLC as follows. The 40% CH_3CN eluate was dried by vacuum centrifugation to remove CH_3CN and lyophilized. The resulting residue was dissolved in ultrapure water and separated using a Shimadzu HPLC system that was equipped with a SPD-20A UV detector (Shimadzu Corp., Kyoto, Japan) and a TSKgel ODS-100V column (4.6 × 150 mm, Tosoh Corporation, Tokyo, Japan). The elution solvents were 0.1% TFA in ultrapure water (A) and 0.1% TFA in CH_3CN (B). The separation conditions were as follows: 0–25 min, 10%–50% B; 25–35 min, 10% B; flow rate, 1 ml/min; and column temperature, 40°C. Elution was monitored at 210 nm, and 2.5 ml fractions were collected. The fractions were dried by vacuum centrifugation to remove CH_3CN and lyophilized. The resulting residue was dissolved in ultrapure water and evaluated for its ability to induce HO-1 expression.

To further purify the active peptides, the most active fractions, fraction (F) 8, were pooled and subjected to a second HPLC separation. F8 (2.4 mg) was injected onto a TSKgel ODS-100V column (4.6 × 150 mm, Tosoh Corporation) with a mixture of 0.1% TFA in ultrapure water (A) and 0.1% TFA in CH_3CN (B), and elution was performed as follows: 0–25 min, 20%–60% B; 25–35 min, 20% B. One milliliter fractions were collected. Each fraction was lyophilized and dissolved in 300 μl ultrapure water. Fifty microliters of each fraction was added to HepG2 cells to induce HO-1 expression. The most active fraction (F8-13) was used for the mass spectrometry analysis for peptide identification.

Mass spectrometry. The peptide sequences of the fraction (F8-13) obtained from HPLC were determined using MALDI-TOF/TOF-MS (UltrafleXtreme, Bruker Daltonics, Bremen, Germany). F8-13 was mixed with a fourth-fold amount of HCCA matrix solution (saturated in CH_3CN/0.1% TFA, 1:2). A mixture of 1 μl was spotted by the dried droplet method on a target plate (MTP 384 target plate ground steel TF, Bruker Daltonics). The washing buffer (0.1% TFA in 10 mM ammonium dihydrogen phosphate) (4 μl) was dropped and air-dried at room temperature (23–25°C). Spectra were processed using Bruker Daltonics Data Analysis and BioTools software. The data were analyzed by a database search using Mascot software (Matrix Science, London, UK).

siRNA transfection of Nrf2. siRNA transfection was conducted as previously described.[23] Silencer select pre-designed siRNA for Nrf2 and negative control siRNA were purchased from Ambion (Life Technologies, Carlsbad, CA). The target sequences for the Nrf2 siRNAs were 5-GAAUGG UCCUAAAACACCATT-3 (sense) and 5-UGGUGUUUUAGG ACCAUUCTG-3 (antisense). Reverse transfection of siRNAs into HepG2 cells was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Each siRNA was diluted with Opti-MEM I Reduced Serum Medium (Gibco, Carlsbad, CA), and Lipofectamine RNAiMAX was added to 6-well plate. After 20 min of incubation at room temperature (23–25°C), a suspension of 2.5×10^5 cells/well was added to obtain a final siRNA concentration of 10 μM. After 48 h of incubation, the medium was replaced with fresh medium with or without 6 μM identified peptide. Cells were harvested for 24 h to measure HO-1 and Nrf2 expression. The efficiency of Nrf2 knockdown was confirmed by real-time PCR.

Statistics. Data are expressed as the mean ± SEM. Statistical analysis of the data was performed using analysis of variance (ANOVA), followed by Dunnett's test to identify differences among groups. Data analysis was performed using Statcel4 software (OMS Publishing Inc., Tokyo, Japan). P values <0.05 were considered to be statistically significant.

Results

Protective effect of RP on H_2O_2-induced oxidative stress. The cytoprotective effects of RP against H_2O_2-induced cell injury were analyzed by measuring LDH leakage. HepG2 cells were pretreated with RP (0, 2.5, 5, and 10 mg/ml) for 24 h and then exposed to H_2O_2 for 24 h. The treatment of control cells with 150 and 300 μM H_2O_2 resulted in cytotoxicity of 26.2 ± 1.1% and 32.5 ± 2.1%, respectively (Fig. 1). However, RP treatment protected cells in a dose-dependent manner. Pretreatment with 5 mg/ml RP reduced the cytotoxicity in of 16.7 ± 1.1% at 300 μM H_2O_2.

Effect of RP on HO-1 and Nrf2 protein expression in HepG2 cells. We assessed the protein expression levels of HO-1 and Nrf2 after RP treatment in HepG2 cells. After treatment of HepG2 cells with RP (0, 2.5, 5, and 10 mg/ml) for 24 h, HO-1 protein expression increased in a dose-dependent manner (Fig. 2A). Protein expression in cells treated with 10 mg/ml RP increased by 2.5 ± 0.1-fold. We further observed the time-dependent effects of RP on HO-1 protein expression. Cells were treated with 5 mg/ml RP at the indicated time points. HO-1

doi: 10.3164/jcbn.21-125

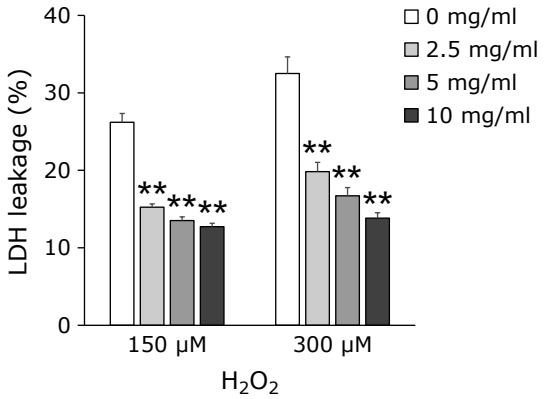

Fig. 1. Protective effect of RP against cell damage caused by oxidative stress in HepG2 Cells. HepG2 cells were treated with RP (0, 2.5, 5, and 10 mg/ml) for 24 h and subsequently exposed to H_2O_2 (150 or 300 μM) for 24 h. The cytotoxicity was determined by measurement of LDH activity released from damaged cells into the medium. Values are the means ± SEM (n = 6). **p<0.01 vs 0 mg/ml.

protein expression decreased at 1 and 3 h. Its protein expression increased significantly (p<0.01) by 2.5 ± 0.2-fold at 8 h (Fig. 2B). HO-1 expression is regulated by Nrf2. Since HO-1 was induced by RP, we decided to examine the Nrf2 expression. Nrf2 protein levels started to increase at 1 h significantly (p<0.01) and further increased by 1.8 ± 0.2-fold at 3 h, which was followed by a gradual decrease to its basal levels (Fig. 2B).

Purification of HO-1 inducer from RP. To identify the HO-1 inducer, we fractionated RP using a Sep-Pak C18 cartridge. The yield of each eluate obtained from 2.3 g of RP was as follows: 0% CH_3CN eluate, 1,609.1 mg; 2.5% CH_3CN eluate, 199.3 mg; 5% CH_3CN eluate, 132.1 mg; 10% CH_3CN eluate, 125.4 mg; 40% CH_3CN eluate, 174.7 mg. Among the five eluates, the 40% CH_3CN eluate significantly (p<0.01) induced HO-1 protein expression (Fig. 3A). We further separated the eluate using reverse-phase HPLC to identify the active compounds. The HPLC chromatogram of the 40% CH_3CN eluate is shown in Fig. 3B. Elution was divided into eight fractions, and 2.5 ml fractions were collected. The yield of each fraction obtained from 86 mg of 40% CH_3CN eluate was as follows: F2, 1.8 mg; F3, 7.9 mg; F4, 17.4 mg; F5, 20.9 mg; F6, 18.6 mg; F7, 11.8 mg; F8, 4.3 mg; F9, 2.6 mg. The activity of each fraction between F2 and F9 is shown in Fig. 3C. Because the F8 significantly (p<0.01) induced HO-1 protein expression, F8 was used for the second purification by reverse-phase HPLC. The second HPLC chromatogram of F8 is shown in Fig. 3D. Ten fractions (F8-10–F8-19 from F8) were collected. The activity of each fraction between F8-10 and F8-19 is shown in Fig. 3E. Among the ten fractions, F8-13 significantly (p<0.01) induced HO-1 protein expression.

The most active fraction (F8-13) was used for MALDI-TOF/TOF-MS analysis for peptide identification. We obtained three sequences from F8-13 (Table 1). The resulting sequences were designated P1, P2, and P3 in the order of their molecular weights. These peptides were chemically synthesized and evaluated for their cytoprotective effects against H_2O_2-induced cell injury. The treatment of control cells with 300 μM H_2O_2 resulted in a cytotoxicity of 29.9 ± 1.8% (Fig. 4). On the other hand, pretreatment with 6 μM P3 reduced the cytotoxicity by 19.9 ± 0.7%. In addition, treatment of HepG2 cells with 6 μM of each peptide for 24 h, P3 significantly (p<0.05) induced HO-1 protein expression

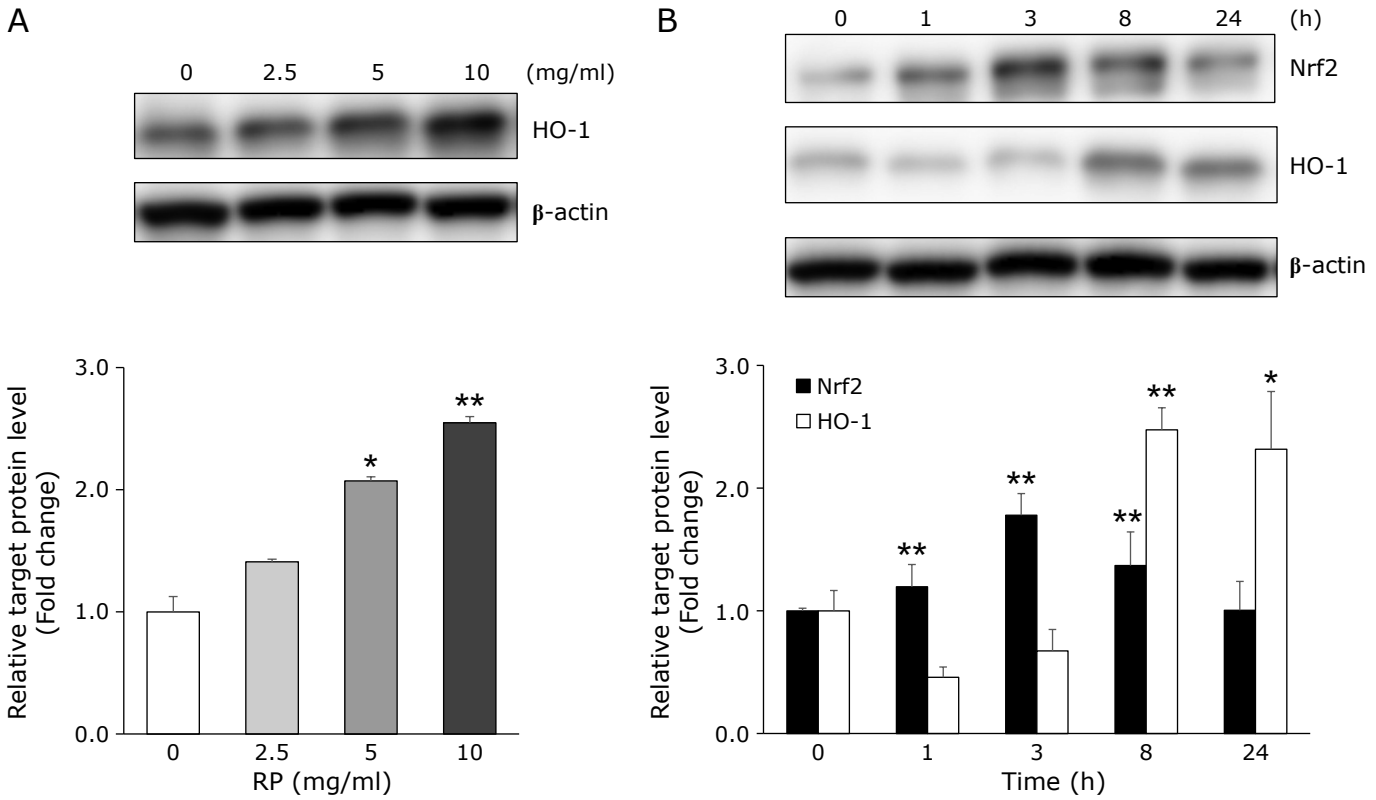

Fig. 2. Effects of RP treatment on HO-1 and Nrf2 expression levels. Protein extracts from HepG2 cells were analyzed by SDS-PAGE and immunoblotting using antibodies against the target proteins. (A) Effect of RP on HO-1 expression levels. The cells were treated with the indicated concentrations of RP for 24 h. Values are mean ± SEM (n = 6). *p<0.05 and **p<0.01 vs 0 mg/ml. (B) Time-dependent effects of RP on HO-1 and Nrf2 expression levels. The cells were treated with 5 mg/ml RP for the indicated times. Values are mean ± SEM (n = 3). *p<0.05 and **p<0.01 vs 0 h.

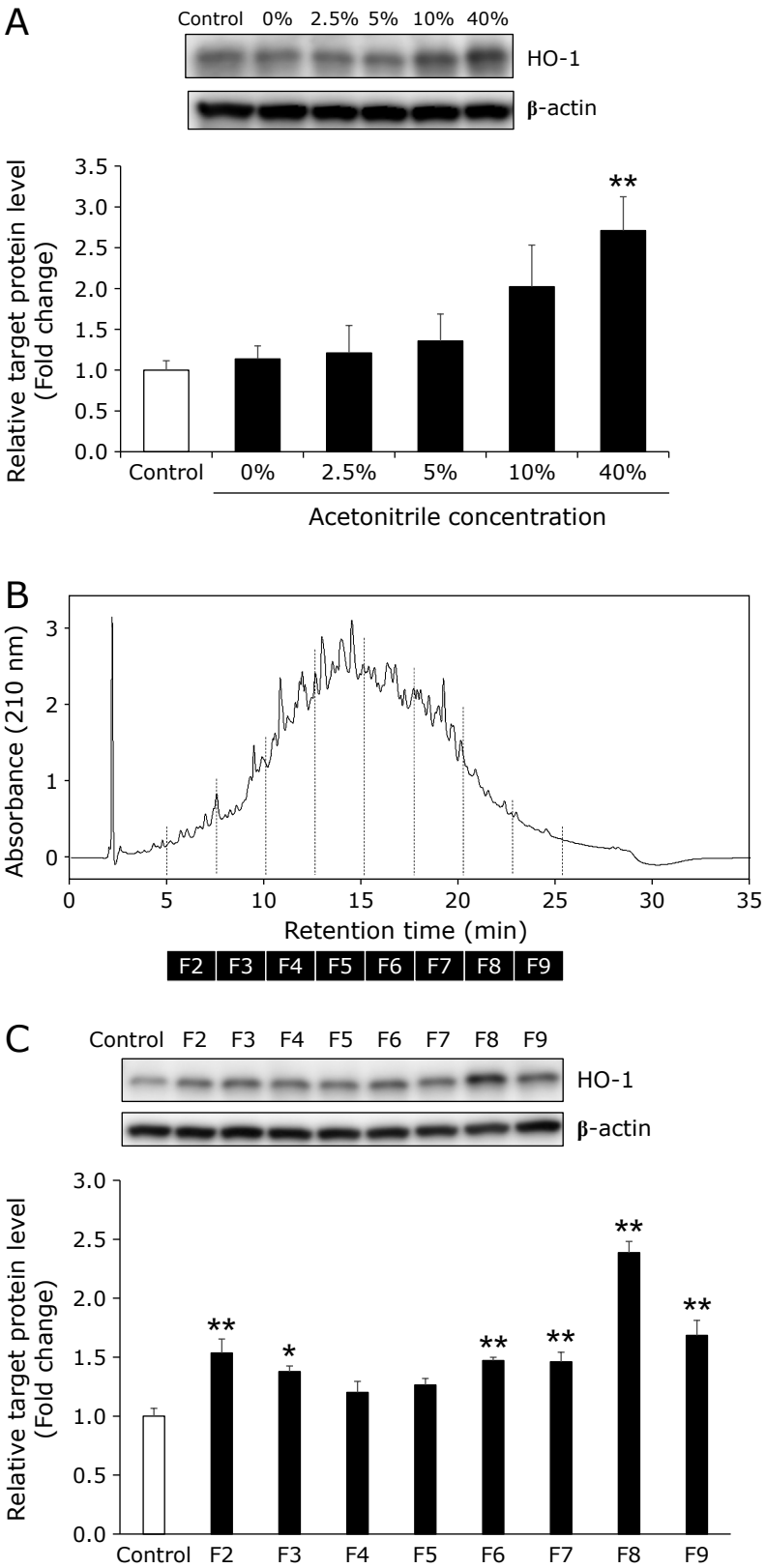

Fig. 3. Effects of HO-1 protein expression by different fractions of RP. Protein extracts from HepG2 cells were analyzed by SDS-PAGE and immunoblotting using antibodies against the target proteins. (A) Fractionation of HO-1 inducers by the Sep-Pak cartridge. Cells were treated with fractions at a concentration of 2.5 mg/ml for 24 h. (B) HPLC chromatogram for the first purification of the active fraction separated using a TSKgel ODS-100V column. (C) Effects of HO-1 protein expression on the fraction of the first HPLC purification. Cells were treated with fractions at a concentration of 0.1 mg/ml for 24 h. (D) HPLC chromatogram for the second purification of the active fraction separated using a TSKgel ODS-100V column. (E) Effect of HO-1 protein expression on the fractions collected during the second HPLC purification. The cells were treated with each fraction for 24 h. Values are mean ± SEM (n = 3). *p<0.05 and **p<0.01 vs control.

doi: 10.3164/jcbn.21-125

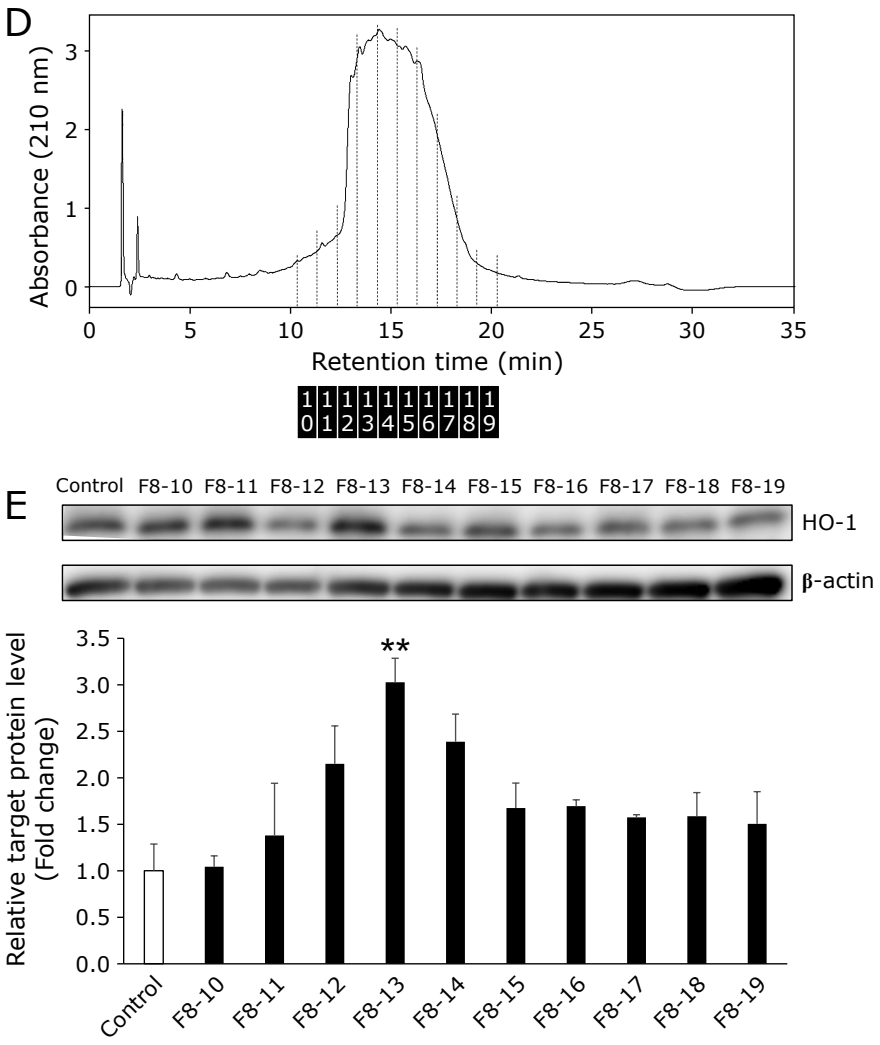

Fig. 3. continued

Table 1. Peptide sequences identified by MALDI-TOF/TOF-MS in active fraction (F8-13) obtained from the second RP-HPLC purification

Peptide	Obs. mass	Calc. mass	Peptide sequence
P1	599.40	598.73	TVAIPV
P2	767.54	767.91	RKKSIH
P3	881.58	882.01	RSAVLLSH

by 2.0 ± 0.1-fold (Fig. 5).

Role of Nrf2 in P3-mediated HO-1 protein expression. To investigate whether P3 induced HO-1 protein expression via Nrf2 activation, we evaluated HO-1 and Nrf2 protein expression after siRNA-mediated *Nrf2* knockdown. *Nrf2* mRNA levels were effectively reduced to 55.4% in cells transfected with Nrf2 siRNA (data not shown). Knockdown of the Nrf2 suppressed the levels of P3-induced HO-1 protein expression (Fig. 6).

Discussion

Recent studies have reported that the induction of HO-1 was involved in the cellular defense system in response to oxidative stress.[25] Several compounds derived from natural products have been shown to induce HO-1 expression to enhance cytoprotection.[26,27] Most of them are polyphenols, and there are few reports

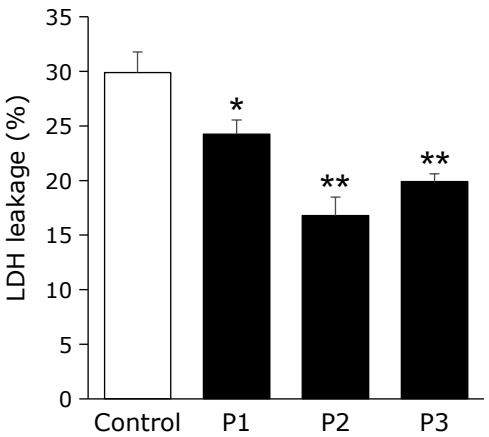

Fig. 4. Protective effect of peptides identified from RP against cell damage caused by oxidative stress in HepG2 Cells. Cells were treated with each peptides (6 μM) for 24 h and subsequently exposed to H_2O_2 (300 μM) for 24 h. The cytotoxicity was determined by measurement of LDH activity released from damaged cells into the medium. Values are mean ± SEM (n = 6). *p<0.05 and **p<0.01 vs control.

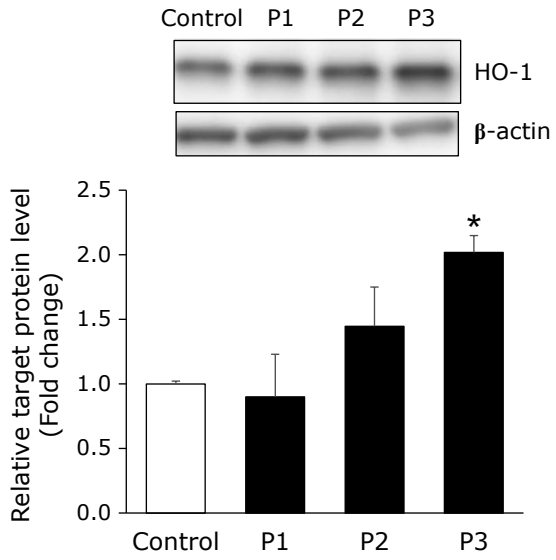

Fig. 5. Effects of peptides identified from RP on HO-1 expression levels. Protein extracts from HepG2 cells were analyzed by SDS-PAGE and immunoblotting using antibodies against the target proteins. Cells were treated with peptides at a concentration of 6 μM for 24 h. Values are mean ± SEM (n = 3). *p<0.05 vs control.

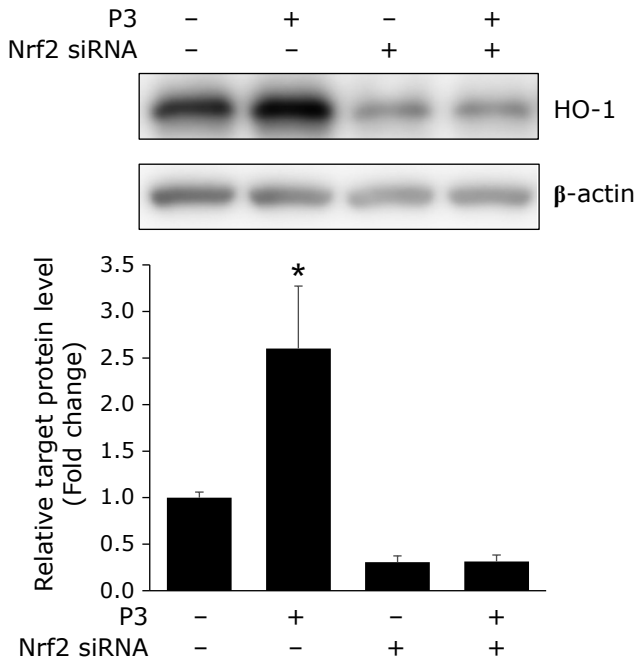

Fig. 6. Effects of *Nrf2* knockdown on HO-1 expression levels. HepG2 cells were transfected with control or *Nrf2* siRNA and incubated for 48 h. After further incubation in fresh medium with or without P3 (6 μM) for 24 h. Protein extracts from cells were analyzed by SDS-PAGE and immunoblotting using antibodies against the target proteins. Values are mean ± SEM (n = 3). *p<0.05 vs control siRNA without P3.

that peptides such as protein hydrolysates induce HO-1 expression. Hydrolysates derived from bovine casein glycomacropeptide,[28] rice bran protein,[29] camel whey protein,[30] and anchovy protein [31] have exhibited cytoprotection against H_2O_2-induced injury by upregulating HO-1 expression. In this study, we demonstrated that RP protected HepG2 cells from H_2O_2-induced cytotoxicity in a dose-dependent manner (Fig.1). We found that pretreatment with RP significantly (p<0.01) upregulated HO-1 expression in a concentration- and time-dependent manner (Fig. 2). Taken together, our results suggest that RP may be able to suppress oxidative stress in cells by increasing HO-1 expression.

Several peptides have been shown to increase the expression of HO-1. For example, the peptide (AMVDAIAR) derived from krill protein hydrolysates exerted a cytoprotective effect against H_2O_2-induced toxicity by increasing the expression of HO-1 in human hepatocytes (Chan hepatocytes).[32] Walnut-derived peptides (LVRL, LRYL, and VLLALVLLR) can increase the expression of HO-1 in HepG2 cells and protect the cells from high glucose-induced insulin resistance and oxidative stress.[33] Peptides (HGSH and KGPSW) derived from edible seahorses have resulted in increased HO-1 expression in H_2O_2-induced human umbilical vein endothelial cells (HUVEC) injury.[34] The three-spot seahorse-derived peptide PAGPRGPA attenuates ethanol-induced oxidative stress in normal human liver cell line (LO2) by promoting the expression of HO-1.[35] In this study, we identified three peptides from the RP through a bioactivity-guided fractionation procedure. The three peptides identified from RP significantly (p<0.05) rescued cells from H_2O_2-induced cell death (Fig. 4). Among them, P3 (RSAVLLSH) significantly (p<0.05) induced HO-1 protein expression (Fig. 5). This peptide, RSAVLLSH, bears no similarity to these peptides derived from krill, seahorse and walnut. Therefore, RSAVLLSH is a novel peptide that induces HO-1 expression. These results suggest that the active peptide with induction of HO-1 expression has a high proportion of hydrophobic amino acid residues and contains one or two basic amino acid residues (His, Arg, and Lys). There was no significant difference in the induction of HO-1 expression between P1 (TVAIPV), which does not contain basic amino acids. Further studies are needed to understand the relationship between the induction of HO-1 expression and amino acids in the peptide sequences.

F8-13 obtained after the second HPLC purification induced three-fold HO-1 protein expression (Fig. 3E), whereas P3 induced two-fold HO-1 protein expression (Fig. 5). In addition, P2 induced slight expression of HO-1 protein. Thus, these results indicate that P3 and P2 in combination may synergistically induce HO-1 protein expression.

HO-1 expression contributes to protection against APAP-induced liver damage.[12] In previous reports, sulforaphane,[36] sake lees hydrolysate,[37] and salvianolic acid C [38] have protective effects against APAP-induced hepatotoxicity through antioxidant effects mediated via HO-1 induction. We previously reported that RP protects against APAP-induced hepatotoxicity in mice.[22] Our results indicate that RSAVLLSH derived from RP may be an active peptide with a hepatoprotective effect via the induction of HO-1 expression.

Under basal conditions, Nrf2 is anchored to Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. Upon stimulation by oxidative stress, Nrf2 dissociates from Keap1 following nuclear translocation and then binds to antioxidant response element (ARE) sites, leading to the activation of ARE-related genes such as HO-1.[39] In our study, P3-induced expression of HO-1 was markedly suppressed by siRNA knockdown of Nrf2 (Fig. 6). These results suggest that P3-induced HO-1 expression requires the activation of the Nrf2 signaling pathway.

In conclusion, we demonstrated that RP protected the cells from oxidative stress induced by H_2O_2 and significantly (p<0.05) induced Nrf2 and HO-1 protein expression. Moreover, we isolated the peptide RSAVLLSH as an HO-1 inducer. This peptide induces the expression of HO-1 by activating the Nrf2 signaling pathway. Our findings indicate that peptides isolated from rice-derived peptides may be useful for the development of functional foods for the prevention of diseases associated with oxidative stress.

doi: 10.3164/jcbn.21-125

Acknowledgments

We acknowledge Professor Taka-aki Miyaji (Advanced Science Research Center, Okayama University, Okayama, Japan) for performing MALDI-TOF/TOF-MS. We thank NAGASE Chemtex Co. Ltd. for providing Denazyme AP and Oryza Oil and Fat Chemical Co. Ltd. for providing Oryza Protein-P70.

Conflict of Interest

No potential conflicts of interest were disclosed.

References

1 Babel RA, Dandekar MP. A review on cellular and molecular mechanisms linked to the development of diabetes complications. *Curr Diabetes Rev* 2021; **17**: 457–473.
2 Cross CE, Halliwell B, Borish ET, *et al*. Oxygen radicals and human disease. *Ann Intern Med* 1987; **107**: 526–545.
3 Gutteridge JM. Free radicals in disease processes: a compilation of cause and consequence. *Free Radic Res Commun* 1993; **19**: 141–158.
4 Ighodaro OM, Akinloye OA. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their fundamental role in the entire antioxidant defence grid. *Alex J Medicine* 2018; **54**: 287–293.
5 Zhang H, Tsao R. Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Curr Opin Food Sci* 2016; **8**: 33–42.
6 Itoh K, Tong KI, Yamamoto M. Molecular mechanism activating Nrf2–Keap1 pathway in regulation of adaptive response to electrophiles. *Free Radic Biol Med* 2004; **36**: 1208–1213.
7 Kobayashi M, Yamamoto M. Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxid Redox Signal* 2005; **7**: 385–394.
8 Kapitulnik J, Maines MD. Pleiotropic functions of biliverdin reductase: cellular signaling and generation of cytoprotective and cytotoxic bilirubin. *Trends Pharmacol Sci* 2009; **30**: 129–137.
9 Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987; **235**: 1043–1046.
10 Brouard S, Otterbein LE, Anrather J, *et al*. Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. *J Exp Med* 2000; **192**: 1015–1026.
11 Poss KD, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. *Proc Natl Acad Sci U S A* 1997; **94**: 10925–10930.
12 Farombi EO, Surh YJ. Heme oxygenase-1 as a potential therapeutic target for hepatoprotection. *J Biochem Mol Biol* 2006; **39**: 479–491.
13 Soares MP, Bach FH. Heme oxygenase-1: from biology to therapeutic potential. *Trends Mol Med* 2009; **15**: 50–58.
14 Takahashi M, Moriguchi S, Yoshikawa M, Sasaki R. Isolation and characterization of oryzatensin: a novel bioactive peptide with ileum-contracting and immunomodulating activities derived from rice albumin. *Biochem Mol Biol Int* 1994; **33**: 1151–1158.
15 Zhang X, Wang L, Wang R, Luo X, Li Y, Chen Z. Protective effects of rice dreg protein hydrolysates against hydrogen peroxide-induced oxidative stress in HepG-2 cells. *Food Funct* 2016; **7**: 1429–1437.
16 Liu K, Zhao Y, Chen F, Fang Y. Purification and identification of Se-containing antioxidative peptides from enzymatic hydrolysates of Se-enriched brown rice protein. *Food Chem* 2015; **187**: 424–430.
17 Zhang X, Wang L, Lu H, *et al*. Preservation of hydrogen peroxide-induced oxidative damage in HepG-2 cells by rice protein hydrolysates pretreated with electron beams. *Sci Rep* 2020; **10**: 8415.
18 Taniguchi M, Matsuhashi Y, Abe TK, *et al*. Contribution of cationic amino acids toward the inhibition of Arg-specific cysteine proteinase (Arg-gingipain) by the antimicrobial dodecapeptide, CL(14-25), from rice protein. *Biopolymers* 2014; **102**: 379–389.
19 Ishikawa Y, Hara T, Inoue D, *et al*. Rice protein hydrolysates stimulate GLP-1 secretion, reduce GLP-1 degradation, and lower the glycemic response in rats. *Food Funct* 2015; **6**: 2525–2534.
20 Hatanaka T, Uraji M, Fujita A, Kawakami K. Anti-oxidation activities of rice-derived peptides and their inhibitory effects on dipeptidylpeptidase-IV. *Int J Pept Res Ther* 2015; **21**: 479–485.
21 Ina S, Hamada A, Nakamura H, Yamaguchi Y, Kumagai H, Kumagai H. Rice (*Oryza sativa japonica*) albumin hydrolysates suppress postprandial blood glucose elevation by adsorbing glucose and inhibiting Na^+-D-glucose cotransporter SGLT1 expression. *J Funct Foods* 2020; **64**: 103603.
22 Kawakami K, Moritani C, Uraji M, *et al*. Hepatoprotective effects of rice-derived peptides against acetaminophen-induced damage in mice. *J Clin Biochem Nutr* 2017; **60**: 115–120.
23 Moritani C, Kawakami K, Shimoda H, Hatanaka T, Suzaki E, Tsuboi S. Protective effects of rice peptide oryza peptide-P60 against oxidative injury through activation of Nrf2 signaling pathway *in vitro* and *in vivo*. *ACS Omega* 2020; **5**: 13096–13107.
24 Morita K, Nishibori N, Kishibuchi R, Itoh M, Horie Y, Nemoto H. Fermented brown rice extract stimulates BDNF gene transcription in C6 glioma cells: possible connection with HO-1 expression. *J Diet Suppl* 2017; **14**: 214–228.
25 Waza AA, Hamid Z, Ali S, Bhat SA, Bhat MA. A review on heme oxygenase-1 induction: is it a necessary evil. *Inflamm Res* 2018; **67**: 579–588.
26 Mehta J, Rayalam S, Wang X. Cytoprotective effects of natural compounds against oxidative stress. *Antioxidants (Basel)* 2018; **7**: 147.
27 Funes SC, Rios M, Fernández-Fierro A, *et al*. Naturally derived heme-oxygenase 1 inducers and their therapeutic application to immune-mediated diseases. *Front Immunol* 2020; **11**: 1467.
28 Li T, Chen B, Du M, *et al*. Casein glycomacropeptide hydrolysates exert cytoprotective effect against cellular oxidative stress by up-regulating HO-1 expression in HepG2 cells. *Nutrients* 2017; **9**: 31.
29 Moritani C, Kawakami K, Fujita A, Kawakami K, Hatanaka T, Tsuboi S. Anti-oxidative activity of hydrolysate from rice bran protein in HepG2 cells. *Biol Pharm Bull* 2017; **40**: 984–991.
30 Du D, Lv W, Su R, *et al*. Hydrolyzed camel whey protein alleviated heat stress-induced hepatocyte damage by activated Nrf2/HO-1 signaling pathway and inhibited NF-κB/NLRP3 axis. *Cell Stress Chaperones* 2021; **26**: 387–401.
31 Giannetto A, Esposito E, Lanza M, *et al*. Protein hydrolysates from anchovy (*Engraulis encrasicolus*) waste: *in vitro* and *in vivo* biological activities. *Mar Drugs* 2020; **18**: 86.
32 Fernando IPS, Park SY, Han EJ, *et al*. Isolation of an antioxidant peptide from krill protein hydrolysates as a novel agent with potential hepatoprotective effects. *J Funct Foods* 2020; **67**: 103889.
33 Wang J, Wu T, Fang L, *et al*. Peptides from walnut (*Juglans mandshurica* Maxim.) protect hepatic HepG2 cells from high glucose-induced insulin resistance and oxidative stress. *Food Funct* 2020; **11**: 8112–8121.
34 Oh Y, Ahn CB, Je JY. Cytoprotective role of edible seahorse (*Hippocampus abdominalis*)-derived peptides in H_2O_2-induced oxidative stress in human umbilical vein endothelial cells. *Mar Drugs* 2021; **19**: 86.
35 Shi J, Zhou X, Zhao Y, *et al*. The three-spot seahorse-derived peptide PAGPRGPA attenuates ethanol-induced oxidative stress in LO2 cells through MAPKs, the Keap1/Nrf2 signalling pathway and amino acid metabolism. *Food Funct* 2021; **12**: 1672–1687.
36 Noh JR, Kim YH, Hwang JH, *et al*. Sulforaphane protects against acetaminophen-induced hepatotoxicity. *Food Chem Toxicol* 2015; **80**: 193–200.
37 Kawakami K, Moritani C, Uraji M, *et al*. Sake lees hydrolysate protects against acetaminophen-induced hepatotoxicity via activation of the Nrf2 antioxidant pathway. *J Clin Biochem Nutr* 2017; **61**: 203–209.
38 Wu CT, Deng JS, Huang WC, Shieh PC, Chung MI, Huang GJ. Salvianolic acid C against acetaminophen-induced acute liver injury by attenuating inflammation, oxidative stress, and apoptosis through inhibition of the Keap1/Nrf2/HO-1 signaling. *Oxid Med Cell Longev* 2019; **2019**: 9056845.
39 Kobayashi M, Yamamoto M. Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. *Adv Enzyme Regul* 2006; **46**: 113–140.