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Casein glycomacropeptide hydrolysate exerts cytoprotection against H_2O_2-induced oxidative stress in RAW 264.7 macrophages via ROS-dependent heme oxygenase-1 expression

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Abstract

The aim of this study was to investigate the antioxidant potential of bovine casein glycomacropeptide (GMP) and its hydrolysate, as well as to determine effects of GMP and its hydrolysate on hydrogen peroxide (H_2O_2)-induced oxidative stress in RAW 264.7 macrophages. In comparison with native GMP, GMP hydrolysate obtained with papain for 1 h hydrolysis (GHP) exerted higher free radicals-scavenging capacity, ferrous ions (Fe^{2+})-chelating ability, and ferric reducing (FRAP) activity. GHP significantly blocked H_2O_2-induced intracellular reactive oxygen species (ROS) generation as well as cell death, and the cytoprotective effects of GHP were partially reversed by co-treatment with zinc (II)-protoporphyrin IX (ZnPPIX), a specific inhibitor of HO-1. GHP induced nuclear translocation of the nuclear factor-erythroid-2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) expression in RAW 264.7 macrophages. The chemical antioxidant, N-acetyl cysteine (NAC), significantly reduced GHP-induced HO-1 expression and Nrf2 activation by blocking intracellular ROS production. Additionally, pretreatment with GHP

enhanced cellular antioxidant enzymes activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) in H_2O_2-damaged cells. The antioxidant activity of GHP may be attributed to its amino acid profiles. Compared with native GMP, GHP had higher contents of alanine, glycine, glutamic acid, aspartic acid and branched chain amino acids (BCAAs, leucine, isoleucine and valine). BCAAs-enriched GHP may possess a potential to ameliorate oxidative stress-related diseases.

1. Introduction

Oxidative stress may be a hallmark of several metabolic diseases, including obesity, non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes mellitus (T2DM).[1-3] Oxidative stress is a condition characterized by excess production of intracellular reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O^{2-}) and hydroxyl radical (·OH) in combination with outstripping endogenous antioxidant defense systems.[4, 5] This oxidative stress can then damage cellular macromolecules (lipids, proteins and DNA) and triggers inflammatory responses associated with adiposity as well as insulin resistance.[6-8] Inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) will in turn induce ROS generation, which leads to exacerbation of oxidative stress.[9]

Macrophages that function as immune sentinels can release various biologically active mediators which regulate inflammation and restore tissue homeostasis.[10, 11] Once localized in tissues, macrophages acquire specialized functions depending on the requirements of the tissue.[12] Long-term excessive ROS production caused by metabolic abnormalities will injure immunocytes, thereby resulting in cell death and loss of function. Furthermore, when continuously activated, macrophages can produce large amounts of O^{2-} and H_2O_2. These ROS exert strong cytotoxic activities against

many cells, including macrophages themselves.[13] Excessive ROS-induced oxidative damage in macrophages which is characterized by impaired responses to tissue damage has been proposed to contribute to the pathogenesis of obesity complications such as infection and impaired tissue healing.[14] Moreover, the functions of macrophages in reverse cholesterol transport can be impaired by oxidative stress.[15] Protective effects against oxidative stress-associated physiological disorders were observed after systemic administration of antioxidants to mice, resulting in reduced oxidative stress in macrophages.[16, 17]

Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and heme oxygenase-1 (HO-1) play a key role in the defence against oxidative stress. Accumulating evidences suggest that upregulation of HO-1 expression which is mediated by activation of the nuclear factor-erythroid-2-related factor 2 (Nrf2) may confer adaptive survival response to oxidative stress both in vivo and in vitro.[18, 19] Antioxidant supplementation may have protective effects on oxidative damage via fortifying cellular antioxidant defense system. Lycopene inhibited the formation of ROS and affected the activities of a battery of oxidant enzymes in the skin of mice.[20] Supplementation of chromium histidinate was protective against oxidative stress in obesity, at least in part, through Nrf2-mediated induction of HO-1 in rats fed high fat diet.[21] Current evidences also indicate that antioxidant activity of dairy protein and derived hydrolysates. All the subunits of casein (α-casein, β-casein and κ-casein) are able to inhibit Fe-induced lipid oxidation in a liposomal model system.[22] Ovine κ-casein-derived peptide was a potent inhibitor of linoleic acid oxidation with an activity similar to that obtained with the synthetic antioxidant BHT.[23] Whey protein hydrolysates on H_2O_2-induced PC12 cells oxidative stress via a mitochondria-mediated pathway.[24] Casein

glycomacropeptide (GMP) is a glycopeptide of 64 amino acid residues derived from κ-casein. GMP and its hydrolysates have deserved much interest for their proposed biological activities, including antibacterial activity and antiapoptotic effect on mice with ulcerative colitis.[25, 26] In addition, a previous study in our laboratory indicated that GMP could counteract high-fat diet-induced obesity by reducing plasma total cholesterol and low-density lipoprotein (LDL) cholesterol as well as hepatic-cholesterol and triglycerides. Meanwhile, leptin production and pro-inflammatory cytokines such as TNF-α and IL-6 secretion also decreased.[27] However, the antioxidant activity and the protective effects of GMP and its derived components on oxidative stress-induced macrophage dysfunction are still unclear. The roles of HO-1 in the protective effects of GMP and its derived components against H_2O_2-induced oxidative damage have not yet been elucidated.

Collectively, these data suggest that increased oxidative stress is an early instigator of metabolic syndrome and that the redox state in macrophages is a potentially useful therapeutic target for oxidative stress-related pathologies. Therefore, the aim of the present study was to evaluate the efficacy of GMP and its hydrolysates for the protection of cells against H_2O_2-induced oxidative damage using RAW 264.7 macrophages as a model system and to analyse the relationship between antioxidant activity and their amino acid profiles. Whether this cytoprotective effect is related to Nrf2 activation and HO-1 expression was also explored.

2. Results and Discussion

2.1 Antioxidant activity of GMP and its hydrolysate

2.1.1 Free radical-scavenging activity of GMP and GHP

GMP hydrolysate obtained with papain for 1 h hydrolysis was named as GHP. The hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2)-scavenging activity of GMP

and GHP were shown in Fig. 1A and 1B. The free radical-scavenging activity of GHP was significantly higher than that of intact GMP at any tested concentration ($P < 0.05$). The highest scavenging capacity of GHP at the concentration of 2.0 mg mL^{-1} for OH radical and H_2O_2 with 67.24% ± 2.60% and 62.05% ± 1.98%, respectively.

2.1.2 Ferrous ions (Fe^{2+})-chelating activities of GMP and GHP

The Fe^{2+}-chelating activity of GMP and GHP was shown in Fig. 1C. Ferrozine can quantitatively form complexes with Fe^{2+}. The complex formation is disrupted in the presence of chelating agents. The Fe^{2+}-chelating activity of GHP was significantly higher than that of intact GMP at any tested concentration ($P < 0.05$). The maximum Fe^{2+} chelating activity of GHP was 75.55% ± 3.51% at the concentration of 2.0 mg mL^{-1}.

2.1.3 Ferric-reducing activity of GMP and GHP

The FRAP values, being expressed as $FeSO_4$ equivalents per mg sample, of GMP and GHP were assayed. The FRAP value of GHP was significantly higher than that of GMP itself ($P < 0.05$). The highest FRAP value of GHP was 185.29 ± 5.19 μM Fe^{2+} at the concentration of 2.0 mg mL^{-1}, as shown in Fig 1D.

Iron toxicity has been presumed to involve the formation of OH radical from H_2O_2 in the Fenton reaction. H_2O_2 itself is not very reactive; however it can sometimes be toxic to cell because it may give rise to OH radical which is the most active ROS.[28] Furthermore, H_2O_2 may induce the increase of iron content in cells and subsequently lead to the deleterious condition.[29] Thus, minimizing Fe^{2+} concentration and removing free radicals may reduce the levels of ROS. Our results demonstrated that GHP showed significant OH radical scavenging activity due to its combined effects of chelating Fe^{2+} and scavenging H_2O_2. Furthermore, FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+}-TPTZ)

126 complex and producing a coloured ferrous tripyridyltriazine (Fe^{2+}-TPTZ). The ferric
127 reducing antioxidant properties associated with the presence of GHP may exert
128 antioxidant action by donating electrons through resonance to stabilize the free
129 radicals.[30] Thus, GHP may afford protection against oxidative damage by direct
130 inhibition of ROS generation in RAW 264.7 macrophages.

131 **2.2 Effect of GHP on H_2O_2-induced ROS accumulation**

132 To understand whether GHP protected cellular damage via inhibiting ROS production,
133 the effect of GHP on cellular ROS level in H_2O_2-stressed RAW 264.7 macrophages
134 was assessed. The production of intracellular ROS was indirectly measured using
135 non-fluorescent dichloro-dihydro-fluorescein diacetate (DCFH-DA), which was
136 converted to fluorescent dichlorofluorescein (DCF) in the presence of ROS.
137 Quantitative analysis of fluorescence intensity revealed that the intracellular ROS
138 levels were significantly increased by H_2O_2 treatment ($P < 0.05$). GHP pretreatment
139 significantly reduced ROS generation induced by H_2O_2 in a dose dependent manner
140 from 0.25 mg mL^{-1} to 2.0 mg mL^{-1}, compared with that in the H_2O_2-damaged group
141 ($P < 0.05$), as shown in Fig. 2A. The inhibitory effect of GHP on ROS generation was
142 significantly higher than that of GMP at each treatment concentration ($P < 0.05$). The
143 intracellular ROS levels were 74.04 ± 3.18, 63.89 ± 3.66, 58.03 ± 2.25 and 53.27 ±
144 2.62 (decreased by 13.59%, 25.35%, 32.20% and 37.76%) at 0.25, 0.5, 1.0 and 2.0 mg
145 mL^{-1} GHP, respectively. The intracellular ROS levels were 81.63 ± 1.43, 79.49 ± 0.99,
146 72.00 ± 1.94 and 63.12 ± 1.96 at 0.25, 0.5, 1.0 and 2.0 mg mL^{-1} GMP, respectively.
147 For the intracellular ROS localization, RAW 264.7 macrophages was observed at
148 ×630 magnification under oil immersion with the laser scanning confocal microscope.
149 As shown in Fig. 2B, the control cells displayed weak green fluorescence, whereas the
150 cells treated with H_2O_2 showed striking green fluorescence, reflecting the increase of

intracellular ROS levels. In contrast, this elevation was almost reversed by GHP pretreatment. These results suggested that GHP was a potential free radical scavenger.

2.3 Protective effects of GHP on H_2O_2-induced cell death in RAW 264.7 macrophages

The effect of GHP on the viability of RAW 264.7 macrophages was analysed by methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. Compared with control group (NC), the cell viability of RAW 264.7 macrophages following exposure to H_2O_2 (H_2O_2 damaged group, MC) was significantly decreased ($P < 0.05$), as shown in Fig. 3A. Assuming that the cell viability of control group (NC) was 100%, the cell viability was decreased to 57.26% ± 1.09% when the cells were exposed to H_2O_2. The cell viability of H_2O_2 treatment for 12 h reduced to approximately 50% of control group. The cytotoxic effects of H_2O_2 on RAW 264.7 macrophages were significantly blocked by pretreatment with different concentrations of GHP ($P < 0.05$). The protective effect of GHP on cell viability was significantly higher than that of GMP at each treatment concentration ($P < 0.05$). The cell viability was increased to 64.59% ± 0.86%, 67.95% ± 1.27%, 75.53% ± 1.97% and 80.70% ± 1.11% at 0.25, 0.5, 1.0 and 2.0 mg mL^{-1} GHP, respectively. The cell viability was increased to 58.64% ± 1.10%, 63.70% ± 1.05%, 68.01% ± 0.63% and 71.53% ± 1.69% at 0.25, 0.5, 1.0 and 2.0 mg mL^{-1} GMP, respectively.

To investigate whether GHP protected against H_2O_2-induced apoptosis, RAW 264.7 macrophages were pretreated with different concentrations of GHP (1.0 and 2.0 mg mL^{-1}) before H_2O_2 exposure. Cell apoptosis was examined by flow cytometry using Annexin V plus PI stain. After exposure to H_2O_2, the percentage of apoptotic cells increased from 8.06% ± 1.47% to 25.04% ± 0.95%, as shown in Fig. 3B. However, the percentage of apoptotic cells was decreased to 10.35% ± 1.02% by

pretreatment with GHP at a concentration of 2.0 mg mL^{-1}. Typical cytometry profiles were shown in Fig. 3C. The percentages of cells positive for PI and/or Annexin V-FITC were reported inside the quadrants.

2.4 Effect of GHP on intracellular antioxidant enzymes in RAW 264.7 macrophages subjected to H_2O_2-induced oxidative stress

An imbalance between the antioxidant system and ROS level results in oxidative stress. To determine whether GHP can protect cellular damage by regulating intracellular antioxidant enzyme system, the effects of GHP on the activities of SOD, CAT and GSH-Px in H_2O_2-stressed RAW 264.7 macrophages were measured. As shown in Fig. 4, compared with normal control group (NC), the activities of SOD, CAT and GSH-Px significantly decreased in the H_2O_2 damaged group (MC) ($P < 0.05$). Upon the treatment with H_2O_2, the activities of SOD, CAT and GSH-Px were reduced to 12.44 ± 0.74, 7.54 ± 0.58 and 8.62 ± 0.67 U mg^{-1} protein, respectively. However, compared with H_2O_2 damaged model control, the SOD activity in response to GHP pretreatment at concentrations of 0.5, 1.0 and 2.0 mg mL^{-1} was increased to 17.97 ± 0.51, 21.47 ± 0.47 and 24.09 ± 0.69 U mg^{-1} protein (increased by 43.22%, 72.59% and 91.48%), respectively. The CAT and GSH-Px activities also significantly increased in a dose-dependent manner when H_2O_2-stressed RAW 264.7 macrophages were pretreated with GHP at the concentration from 0.25 mg mL^{-1} to 2.0 mg mL^{-1}.

Oxidative stress reflects an imbalance between the production of ROS and the removal rate of ROS by the antioxidant system, resulting in impaired cell survival thereby affecting cell functions. Macrophage dysfunction induced by oxidative stress may predispose to the development of infection and inflammatory response.[31, 32]

Antioxidant enzymes constitute one of the major cellular protective mechanisms against oxidative injury. SOD is a potent protective enzyme that can catalyse the dismutation of O^{2-} into H_2O_2 and O_2. The other antioxidant enzymes including GSH-Px and CAT can catalyse the conversion of H_2O_2 to water.[33] The present study demonstrated that GHP which exerted high antioxidant activity protected against H_2O_2-induced ROS generation and associated damage to cell survival. Decreased activities of CAT, SOD and GSH-Px caused by oxidative stress were also reversed by the addition of GHP. GHP may exert its protective effects against H_2O_2-induced oxidative damage by scavenging free radicals, Fe^{2+} chelation, and promoting the activity of intracellular antioxidant enzymes.

2.5 Involvement of HO-1 in cytoprotective effect of GHP

2.5.1 Attenuation of the protective effect of GHP against H_2O_2-induced cytotoxicity by the HO-1 enzyme inhibitor, ZnPPIX

HO-1 is one of the main components of cellular antioxidant defense system. To determine whether expression of HO-1 is directly related to cell protection against oxidative damage, RAW 264.7 macrophages were treated with H_2O_2 and GHP in the presence of zinc (II)-protoporphyrin IX (ZnPPIX, a selective inhibitor of HO-1). The changes of cell viability and cellular ROS levels were subsequently measured. As shown in Fig. 5A and 5B, ZnPPIX restored the GHP-mediated suppression of ROS production and elevation of cell viability in H_2O_2-stimulated RAW 264.7 macrophages, while ZnPPIX alone did not exert antioxidant activity. Furthermore, hemin which is a potent inducer of HO-1 also ameliorated H_2O_2-induced oxidative

stress.

2.5.2 GHP-induced HO-1 expression via a ROS-dependent manner in RAW 264.7 macrophages

To further determine whether induction of HO-1 gene expression by GHP was regulated at transcriptional level, the effect of GHP on HO-1 mRNA expression was determined. As shown in Fig. 6A and 6B, HO-1 mRNA levels were gradually upregulated in a dose- and time-dependent manner following exposure to GHP. HO-1 mRNA expression was also significantly increased after treatment with hemin.

Inducible HO-1 is expressed as an adaptive response to several stimuli, including ROS, heme, cytokines and heavy metals et al. In the present culture system, a mild but significant increase in intracellular ROS levels was detected in GHP-treated RAW 264.7 macrophages. However, the addition of N-acetylcysteine (NAC), an ROS scavenger, significantly reduced intracellular ROS production induced by GHP, as shown in Fig. 6C. Strongly elevated levels of intracellular ROS in H_2O_2-treated macrophages was used as a positive control. Additionally, increases in HO-1 mRNA expression and protein level induced by GHP were significantly blocked by the addition of NAC, as shown in Fig. 6D and 6E. These data indicate that ROS induction was involved in GHP-induced HO-1 expression in RAW 264.7 macrophages.

Cells have evolved numerous pathways to counter oxidative damage including activation of stress-related protein responses. Among these, HO-1 which is an inducible stress protein provides antioxidant properties and plays an important role in protection against oxidative insult in chronic disease.[34] Most importantly, HO-1 can

also be induced by various antioxidants to prevent oxidative damage.[35, 36] Therefore, compounds that can induce HO-1 expression may be beneficial in the treatment of oxidative damage. The present study demonstrated that GHP induced HO-1 gene transcription to counteract H_2O_2-induced cellular injury without cytotoxicity in RAW 264.7 macrophages. While high levels of ROS production may result in oxidative stress, increasing evidence demonstrates that moderate levels of ROS may function as signaling molecules in the maintenance of physiological functions.[37] Pretreatment of cells with cytoprotective agents may cause mild oxidative stresses in cells which are sufficient to initiate the intracellular signaling and consequently induce phase II enzyme genes including HO-1 to ameliorate oxidative damage.[38] Epigallocatechin-3-gallate (EGCG), which possesses ROS-scavenging (antioxidant) activity, may cause mild increases in ROS production (pro-oxidant activity) in order to upregulate HO-1 expression.[39] Peptides and amino acids possess both antioxidant and pro-oxidant activity, depending on certain conditions.[40] In the present culture system, it was determined that GHP incubation enhanced intracellular ROS levels in accordance with stimulation of HO-1 expression, and NAC treatment inhibited GHP-induced HO-1 expression by reducing intracellular ROS production.

2.7 GHP induced Nrf2 nuclear translocation via a ROS-dependent manner in RAW 264.7 macrophages

To further elucidate whether GHP induced HO-1 expression via Nrf2 activation, the nuclear level of Nrf2 protein in RAW 264.7 macrophages was analyzed by western blotting and histone H4 was used as internal control. As shown in Fig. 7A, when

RAW 264.7 macrophages were incubated with GHP for 3 h, 6 h, 9 h and 12 h at the concentration of 2.0 mg mL^{-1}, Nrf2 protein level in the nucleus significantly increased with the incubation period. GHP induced Nrf2 nuclear translocation, and thus initiated downstream gene transcription. Additionally, it was determined that GHP-induced Nrf2 nuclear translocation could be suppressed by pretreating RAW 264.7 macrophages with NAC, which suggested that ROS was essential for GHP-induced Nrf2 activation, as shown in Fig. 7B.

Nrf2 is an indispensable regulator of the coordinated induction of phase II enzyme genes including HO-1. The Nrf2 is normally found in the cytoplasm bound to the inhibitory protein. On stimulation, Nrf2 translocates to the nucleus and activates target gene responsible for cytoprotection (the protection of cells from oxidative damage).[41] Procyanidin B2 induces Nrf2 translocation and protects human colonic cells against oxidative stress induced by t-BOOH.[42] The present results demonstrated that the nuclear level of Nrf2 protein increased with incubation time in RAW 264.7 macrophages subjected to GHP, and ROS might participate in GHP-induced Nrf2 nuclear translocation. Generally, these data imply that prevention of H_2O_2 induced-oxidative damage by GHP may be through its direct antioxidant activity or an indirect effect via induction of HO-1 expression by Nrf2 activation in a ROS-dependent manner.

2.8 Amino acid profiles of GMP and GMP hydrolysate (GHP)

To further explore the reason that GHP exerts cellular antioxidant effects, the amino acid profiles of native GMP and GHP were determined, as shown in Table 1. GMP

was hydrolysed by papain for 1 h and then centrifuged at 4000 g for 20 min to remove unhydrolysed protein. The contents of alanine (Ala), glycine (Gly), lysine (Lys), aspartic acid (Asp), glutamic acid (Glu) and branched chain amino acids (BCAAs) including leucine (Leu), isoleucine (Ile) and valine (Val) in GHP were higher than those in native GMP.

The antioxidant activity of food proteins can be attributable to their special amino acid profiles. BCAAs, which are essential nutrients that the body obtains from proteins in food, exert an impressive protection effect against oxidative stress. Animal study has suggested supplementation with BCAAs was associated with reduced oxidative stress and improved glucose metabolism in rats with liver cirrhosis.[43] In addition to BCAAs, Ala and Gly also played a protective role against oxidative stress-induced cell damage by elevating the activities of antioxidant enzymes such as HO, SOD and CAT.[44, 45] Pea seed protein hydrolysates with the best antioxidant properties had the highest Glu and Asp contents.[46] Furthermore, the carboxyl (C=O) groups of Asp and Glu were often involved in chelation of toxic metals responsible for the generation of OH radical.[47] Therefore, it is believed that peptides with high contents of these amino acids can enhance cellular antioxidant activity. The data in the present study demonstrated that GHP enriched with BCAAs, Ala, Asp, Glu and Gly protected macrophages from oxidative damage by scavenging ROS and activating antioxidant mechanisms.

3. Experimental

3.1 Materials

GMP (GMP content of protein is minimum 95%) was provided by Arla Co. (Sønderhøj, Denmark). Papain (EC 3.4.22.21, with activity of 2 U mg^{-1}), Methylthiazolyldiphenyl-tetrazolium bromide (MTT), Dimethyl sulphoxide (DMSO), 2, 4-dinitrophenylhydrazine (DNPH), guanidine hydrochloride, dichloro-dihydro-fluorescein diacetate (DCFH-DA), N-acetylcysteine (NAC), hemin and zinc (II)-protoporphyrin IX (ZnPPIX) were obtained from Sigma-Aldrich (St Louis, MO, USA). The bicinchoninic acid (BCA) protein assay kit was obtained from Pierce (Rockford, IL, USA). Primary rabbit monoclonal HO-1 and Nrf2 antibodies were purchased from Cell Signaling Technology (CST, Beverly, MA). Anti-actin antibody, anti-Histone H4 antibody and horseradish peroxidase-conjugated anti-species (mouse and rabbit) secondary antibodies were purchased from Beyotime Biotech (Haimen, Jiangsu, China).

3.2 Preparation of GMP hydrolysate (GHP)

GMP was dissolved in distilled water at a concentration of 5% (w/v). The protein solutions were adjusted to the optimum pH and temperature for the enzymatic hydrolysis process. Afterward, the protein solution was hydrolysed by papain for 1 h at an enzyme to protein ratio of 5% (w/w, defined as enzyme mass/substrate mass× 100%) under the optimal condition (pH 6.0, 55 °C) as recommended by the manufacturer. During the hydrolysis process, 1 M NaOH solution was continuously added to maintain the optimal pH. After hydrolysis, GMP hydrolysate were collected and immediately heated at 85 °C in a water bath for 20 min to inactivate the protease and stop the hydrolysis reaction. When they were cooled to room temperature, the

hydrolysate solutions were centrifuged at 4000 g for 20 min to remove unhydrolysed protein. The supernatants were collected and lyophilized.

3.3 Determination of antioxidant activity of GMP and GHP

3.3.1 Hydroxyl radical (·OH)-scavenging activity

The OH radical-scavenging activity of GMP and GHP was measured according to the method of Yu *et al.*[48] In this system, OH radical were generated by the Fenton reaction. Briefly, typical reactions were started by addition of 1 mL phenanthroline alcohol solution (0.75 mM) to a tube containing 2 mL PBS (0.2 mM), 1 mL deionized water, 1 mL $FeSO_4$ (0.75 mM) and 1 mL H_2O_2 solution (0.01%). After incubation for 60 min at 37 °C, the absorbance of reaction mixture was measured at 536 nm. The radical scavenging rate (%) was calculated according to the following formula:

Hydroxyl radical scavenging rate (%) = (AS-AP)/(AB-AP)×100

Where, AB is the absorbance of the blank, AP is the absorbance of the control and AS is the absorbance in the presence of tested sample.

3.3.2 Hydrogen peroxide (H_2O_2)-scavenging activity

The H_2O_2-scavenging activity of GMP and GHP was determined by the method of Yen and Chung.[49] A total of 1 mL of sample solution at different concentration was mixed with 400 μL H_2O_2 solution (4 mM) and incubated for 20 min at room temperature. The mixture was supplemented with 600 μL HRPase-phenol red solution (300 μg mL^{-1} of HRPase and 4.5 mM phenol red in 100 mM PBS). After incubation for 10 min, the absorbance was measured at 610 nm by a spectrophotometer. The radical scavenging rate (%) was calculated according to the following formula:

353 Hydrogen peroxide scavenging rate (%) = (A0-A1)/A0×100

354 Where, A0 is the absorbance of the control and A1 is the absorbance in the presence
355 of tested sample.

356 **3.3.3 Fe^{2+}-chelating activity**

357 The Fe^{2+}-chelating activity of GMP and GHP was estimated by the method of Carter
358 *et al.*[50] Briefly, one milliliter of different concentrations of sample solution was added
359 to a solution of 2 mM $FeCl_2$ (50 μL). The reaction was initiated by the addition of 0.5
360 mM ferrozine (0.5 mL). After incubated at 30 °C for 10 min, the absorbance of the
361 solution was measured spectrophotometrically at 562 nm. The percentage of
362 inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula
363 given below:

364 Fe^{2+}-chelating activity (%) = (A0- A1)/A0 ×100

365 Where, A0 is the absorbance of the control and A1 is the absorbance in the presence
366 of tested sample. $FeCl_2$ and ferrozine complex formation molecules are present in the
367 control.

368 **3.3.4 Ferric-reducing antioxidant power (FRAP) assay**

369 The 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ, Sigma, St Louis, MO, USA) solution
370 (40 mM HCl as solvent) was mixed with the same volume of 20 mM $FeCl_3 \cdot 6\ H_2O$ and
371 ten times higher volume of acetate buffer (3.1 g sodium acetate and 16 mL acetic acid
372 per litre). The mixture was incubated at 37 °C before use. A portion (2 mL) of
373 Fe^{3+}-TPTZ mixture and 50 μL tested samples (or distilled water for blank) were
374 incubated at 37 °C for 30 min. The absorbance of the samples at 593 nm was recorded

using a colorimetric UV/Vis spectrophotometer (Unico UV-2600, Shanghai, China). The antioxidant activity of tested samples was expressed as FRAP value which was calculated based on a $FeSO_4$ standard curve (prepared with 0-320 μM $FeSO_4 \cdot 7H_2O$) and expressed as μM of $FeSO_4$ equivalent.

3.4 Cell viability analysis

Murine macrophage-like RAW 264.7 cells were obtained from American Type Culture Collection (Manassas, VA, USA). They were cultured at 37 °C with 5% carbon dioxide (CO_2) in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS, Bioin, Israel), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Invitrogen, Carlsbad, CA, USA). H_2O_2 was freshly prepared from 30% stock solution prior to each experiment.

Cell survival was measured by MTT assay on the basis of the succinate dehydrogenase mitochondrial activity. In brief, RAW 264.7 macrophages were seeded onto 96-well plates at the density of 5×10^4 cells per well and grown to reach 50%–60% confluence. Tested samples were diluted in K/Na phosphate buffer (PBS, pH 7.4). The cells were then preincubated with different concentrations of tested samples (0.25, 0.5, 1.0, 2.0 mg mL^{-1}, final concentration in cell plate) for 12 h before exposure to H_2O_2 (250 μM, 12 h). Oxidatively stressed cells in the presence or absence of tested samples were subsequently treated with MTT solution (5 mg mL^{-1}). After 4 h of incubation at 37 °C, the reaction was terminated and the formed formazan was solubilized in DMSO (150 μL). The absorbance of each well was measured at 570 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). Cells treated with PBS alone were set as the control group. Cell viability (%) was expressed as the optical density ratio of each treatment group to the control group. Viability of the

399 control group (PBS only) was taken as 100%.

400 **3.5 Annexin V-FITC/PI apoptosis assay**

401 The cell apoptosis rate was evaluated by Annexin V-FITC apoptosis detection kit
402 (eBioscience, San Diego, CA, USA). Briefly, the cultured RAW 264.7 macrophages
403 were harvested by trypsinization and resuspended in 1X Annexin V binding buffer at
404 a concentration of 10^6 cells mL^{-1}. The cells were then stained with 5 μL Annexin
405 V-FITC and 5 μL PI in the dark and subsequently analysed with flow cytometer (BD
406 Bioscience, San Jose, CA, USA).

407 **3.6 Cellular reactive oxygen determination**

408 Intracellular ROS level was assessed using DCFH-DA as fluorescent label. RAW
409 264.7 macrophages were seeded at a density of 2×10^5 cells per well in a 96-well
410 culture plate. The cells were treated with H_2O_2 after being pretreated with or without
411 tested samples for 12 h. After incubation, the cells were washed with phosphate
412 buffered saline (PBS, pH 7.4). DCFH-DA (final concentration 50 μM) diluted in
413 DMEM without phenol red was added to the cells and then incubated for 60 min at
414 37 °C. Subsequently, the cells were washed three times with PBS to remove
415 extracellular DCFH-DA, and then subjected to fluorescence intensity analysis using a
416 fluorescence plate reader (Fluoroskan Ascent, Thermo Electron Corporation, Milford,
417 MA, USA) at wavelengths of 488 nm for excitation and 520 nm for emission. The
418 intracellular DCF fluorescence in cell samples relative to unstained cells indicated the
419 relative levels of ROS present in the cells. ROS production was imaged using laser
420 scanning confocal microscopy (Zeiss LSM780, Oberkochen, Germany).

421 **3.7 Analysis of cellular antioxidant enzymes activity**

422 Whole cell lysates were prepared with cell lysis buffer as described in section 3.7.
423 SOD, CAT and GSH-Px activity were determined using the commercial assay kits

(Jiancheng Biochemical, Inc., Nanjing, China). In brief, superoxide anion can convert a tetrazolium salt to a formazan dye. Addition of SOD to this reaction reduced O^{2-} levels, thereby lowering the rate of formazan dye formation. SOD activity was measured as the percent inhibition of the rate of formazan dye formation. One unit of SOD was defined as the amount of SOD required for 50% inhibition of the formazan formation. CAT activity was determined based on alteration of H_2O_2 optical density, depending on enzymatic decomposition of H_2O_2 (by the effect of CAT in the sample). One unit of CAT was defined as the decomposition of 1 μM H_2O_2 per second. GSH-Px activity was determined by the reduction of GSSG formed via the NADPH-glutathione reductase system as a continuous indicator system. Loss of NADPH was monitored continuously at 340 nm. The activity of GSH-Px was defined as the decrease of 1 μM GSH per minute and mg protein. The activities of enzymes were expressed as U mg^{-1} protein of the sample.

3.8 Real time quantitative polymerase chain reaction (RT-qPCR)

RAW 264.7 macrophages (1×10^6 cells) were incubated with tested samples or PBS at 37 °C. Total cellular RNA was isolated using Trizol reagent (Tiangen Biotech, Beijing, China). The total RNA (3 μg) was converted to cDNA in a 25 μL reaction volume using TIANScript RT Kit (Tiangen Biotech), according to the manufacturer's instructions. Quantitative RT-PCR was carried out with a Techne Quantica real-time PCR detection system (Techne, Staffordshire, UK) using a SYBR Premix Ex Taq RT-PCR kit (Takara, Otsu, Shiga, Japan) to detect double stranded DNA synthesis. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was served as the internal control. Primer sequences used for amplifications were as follows:5 ′ -CAGAAGAGGCTAAGACCGCCTT-3 ′ (sense) and 5 ′ -TCTGGTCTTTGTGTTCCTCTGTCA-3' (antisense) for heme oxygenase-1 (HO-1);

5′-TGGCAAAGTGGAGATTGTTGC-3′ (sense) and 5′-AAGATGGTGATGGGCTTCCCG-3′ (antisense) for GAPDH. Real time quantitative PCR was performed starting with a denaturation at 95 °C for 120 s followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, 72 °C for 30 s. The SYBR green fluorescence was read at the end of each extension step (72 °C). The specificity of the amplified PCR products was assessed by a melting curve analysis. Analysis of the data was performed by the ΔΔCt method using GAPDH for normalisation of the samples.[51] The results were presented as fold change relative to the control cells.

3.9 Western blotting

The cells were harvested with cell lysis buffer (Beyotime, Haimen, Jiangsu, China) containing 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich). Equal amounts of protein samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). These membranes were blocked with 5% skim milk in PBS-T solution (0.05% Tween-20 in 1 × PBS solution) at room temperature for 2 h and subsequently incubated overnight with the appropriate primary antibody at 4 °C. After washing with PBS-T solution, the PVDF membranes were incubated with peroxidase conjugated secondary antibody for 1 h. Immunolabeled proteins were detected using an immobilon western chemiluminescent HRP substrate (ECL, Millipore).

3.10 Amino acid analysis

The amino acid compositions of the tested samples were analyzed by the method of Bidlingmeyer *et al*.[52] The samples were incubated with 6 N HCl and heated at 110 °C for 24 h in oil bath. Internal standard was added to the mixture. After derivatisation

with phenylisothiocyanate (PITC), the PITC-amino acids were identified and quantified by reversed-phase high-performance liquid chromatography (RP-HPLC; Shimadzu Co., Tokyo, Japan).

3.11 Statistical analysis

The data were expressed as means ± standard deviations (SD). The differences among the groups were analysed by one-way analysis variance (ANOVA) followed by Tukey's method. $P < 0.05$ was considered statistically significant.

4. Conclusion

This work demonstrated that GHP could attenuate H_2O_2-induced oxidative stress injury in macrophages. The protective effects of GHP partly depended on the combination of alleviating intracellular ROS production and restoring the activities of endogenous antioxidants. GHP ameliorated H_2O_2-oxidative damage by induction of HO-1 expression and Nrf2 activation via a ROS dependent manner. Furthermore, the antioxidant effects of GHP had a great relationship with the content of BCAAs, Ala, Gly, Lys, Asp and Glu. Therefore, GHP has the potential to be developed as a nutraceutical antioxidant for health promotion and prevention of oxidative stress-induced diseases.

Acknowledgments

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Captions

Figure 1 Hydroxyl radical (•OH)-scavenging capacity (A), hydrogen peroxide (H_2O_2) scavenging capacity (B), Fe^{2+}-chelating activity (C), and ferric reducing ability (D) of GMP and GHP. All measurements are expressed as means±SD of three separate determinations.

Figure 2 Inhibitory effect of GHP on H_2O_2-induced production of intracellular ROS. RAW 264.7 macrophages were incubated in the presence or absence of H_2O_2. (A) The ROS levels in the macrophages were determined using fluorescence plate reader. The y axis of the ROS fluorescence represented the intensity of the fluorescent DCF in cell samples relative to unstained cells. The results are the means±SD of four independent experiments. Bars with different alphabets are significantly different ($P < 0.05$). NC: control group, MC: H_2O_2 damaged group. (B) The ROS levels were monitored with laser scanning confocal microscope.

Figure 3 Effect of GHP on the viability and apoptosis rate of RAW 264.7 macrophages subjected to H_2O_2-induced oxidative stress. RAW 264.7 macrophages were pretreated with or without GHP at the indicated concentrations. (A) Cell viability was determined by an MTT assay. Viability of the control cells (PBS only) was taken as 100% (means±SD). (B) The cell apoptosis rates. Cellular apoptosis was assayed by Annexin V-FITC and PI counter staining and analyzed with flow cytometry. Bars with different alphabets are significantly different ($P < 0.05$). (C) The original flow cytometry figures. NC: control group, MC: H_2O_2 damaged group.

Figure 4 Effect of GHP on CAT (A), SOD (B) and GSH-Px (C) activity in RAW 264.7 macrophages subjected to H_2O_2-induced oxidative stress. RAW 264.7 macrophages were incubated in the presence or absence of H_2O_2. All measurements are expressed as means±SD of three separate determinations. Bars with different

alphabets are significantly different ($P < 0.05$). NC: control group, MC: H_2O_2 damaged group.

Figure 5 Involvement of HO-1 in GHP-mediated antioxidant stress. (A) RAW 264.7 macrophages were treated with 2.0 mg mL^{-1} GHP and ZnPPIX prior to the addition of H_2O_2 and further incubated for 12 h. Cell viability was measured by MTT assay. Viability of the control group (PBS only) was taken as 100%. Bars with different alphabets are significantly different ($P < 0.05$). NC: control group, MC: H_2O_2 damaged group. (B) RAW 264.7 macrophages were treated with 2.0 mg mL^{-1} GHP and ZnPPIX prior to the addition of H_2O_2 and further incubated for 1 h. ROS level was monitored with fluorescence microscopy.

Figure 6 Effect of GHP on HO-1 expression in RAW 264.7 macrophages. (A) RAW 264.7 macrophages were cultured with various concentrations of GHP for 12 h. (B) RAW 264.7 macrophages were cultured with GHP (2.0 mg mL^{-1}) for different periods. (C) GHP-induced ROS production in RAW 264.7 macrophages. Cells were treated with NAC (10 mM) for 30 min prior to incubation with GHP (2.0 mg mL^{-1}) for 6 h. The ROS levels in the macrophages were determined using fluorescence plate reader. (D) Prevention of GHP-induced HO-1 mRNA expression by NAC. Cells were treated with NAC (10 mM) for 1 h prior to incubation with GHP (2.0 mg mL^{-1}) for 12 h. The data is reported as the means±SD of four experiments performed independently. Bars with different alphabets are significantly different ($P < 0.05$). (E) Prevention of GHP-induced HO-1 protein expression by NAC. Cells were treated with NAC (10 mM) for 1 h prior to incubation with GHP (2.0 mg mL^{-1}) for 12 h. The level of HO-1 protein in RAW 264.7 macrophages was analyzed.

Figure 7 Effect of GHP on Nrf2 activation in RAW 264.7 macrophages. (A) RAW 264.7 macrophages were cultured with GHP (2.0 mg mL^{-1}) for different periods. The

nuclear level of Nrf2 protein in RAW 264.7 macrophages was analyzed. (B) Prevention of GHP-induced Nrf2 activation by NAC. Cells were treated with NAC (10 mM) for 1 h prior to incubation with GHP (2.0 mg mL^{-1}) for 12 h. The nuclear level of Nrf2 protein in RAW 264.7 macrophages was analyzed.

Table 1 Analysis of amino acid profiles of casein glycomacropeptide (GMP) and its hydrolysate (GHP)

Figure 1

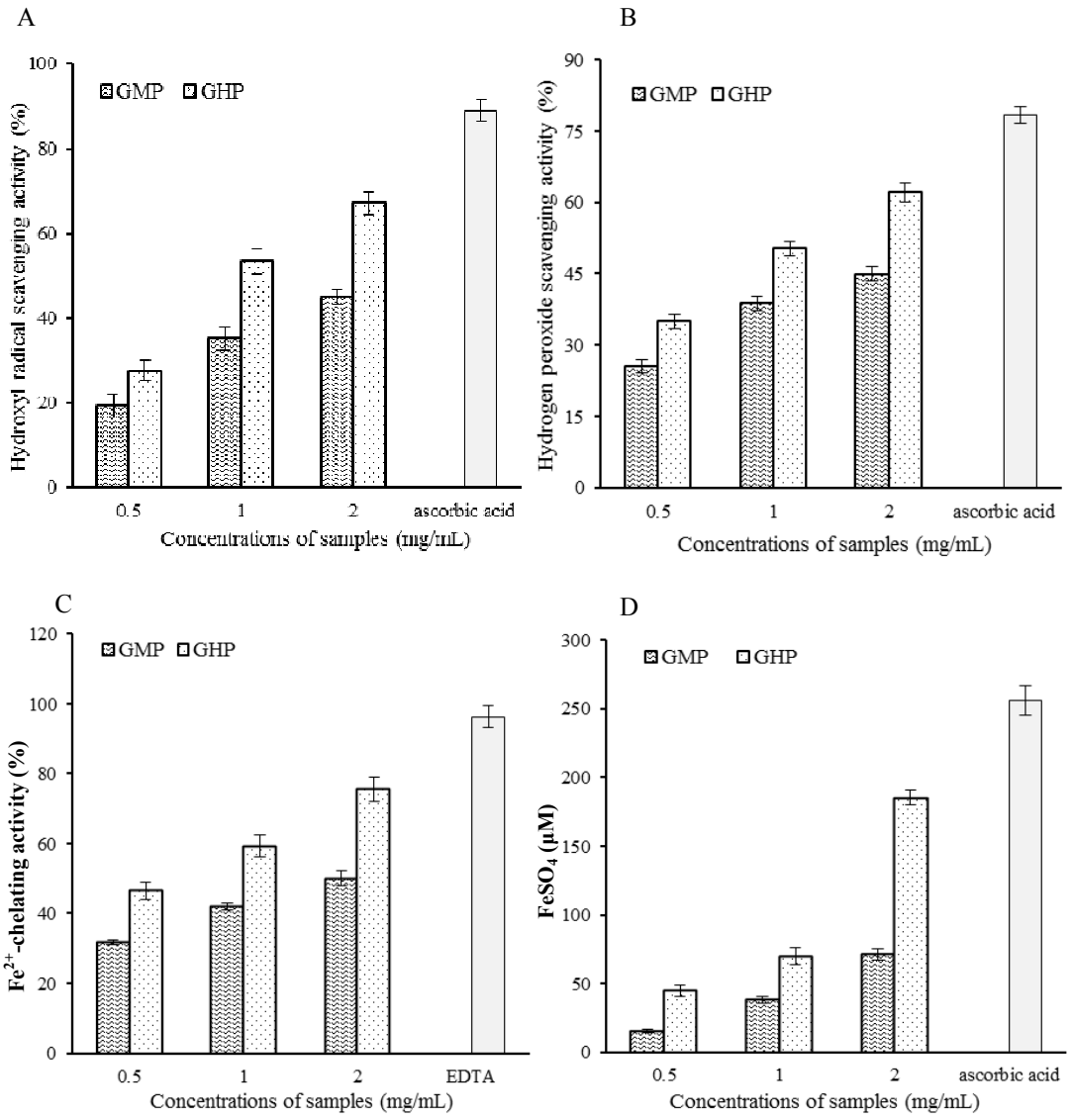
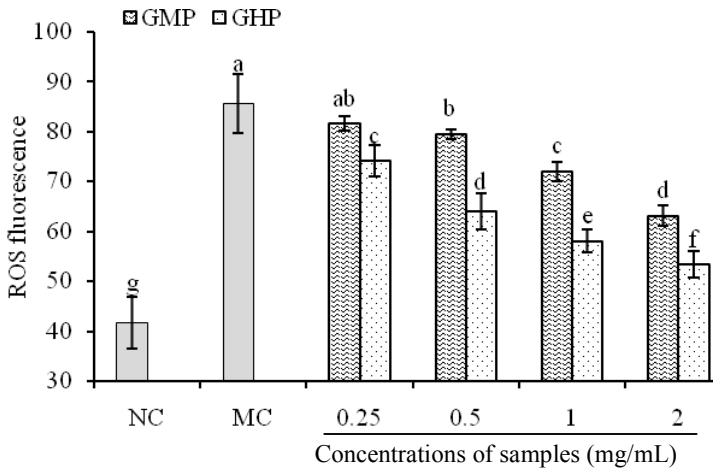


Figure 2

A



B

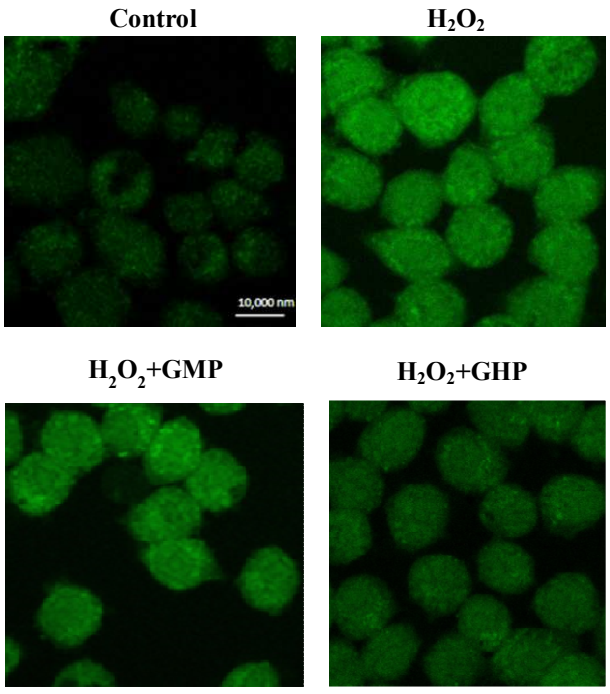


Figure 3

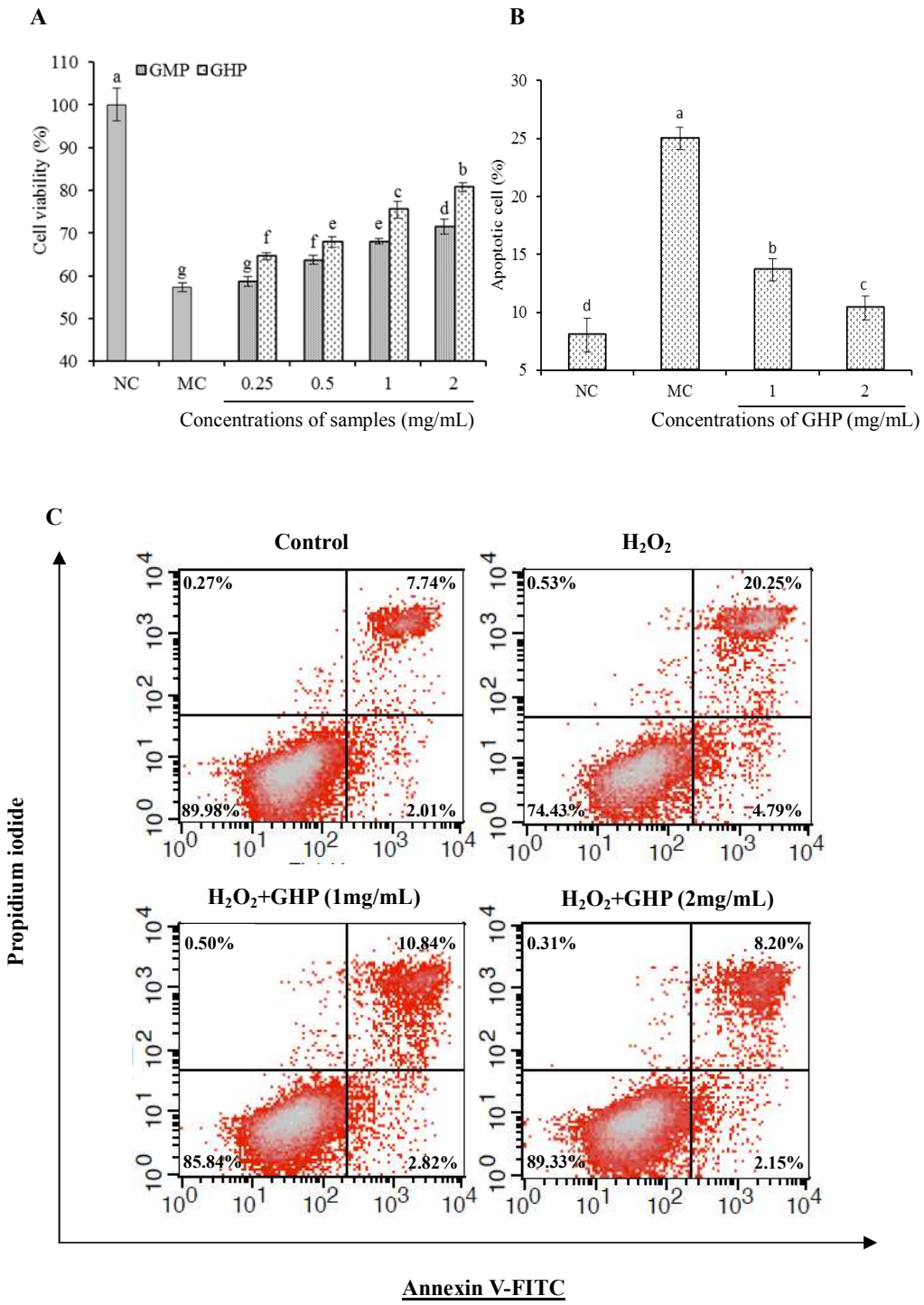


Figure 4

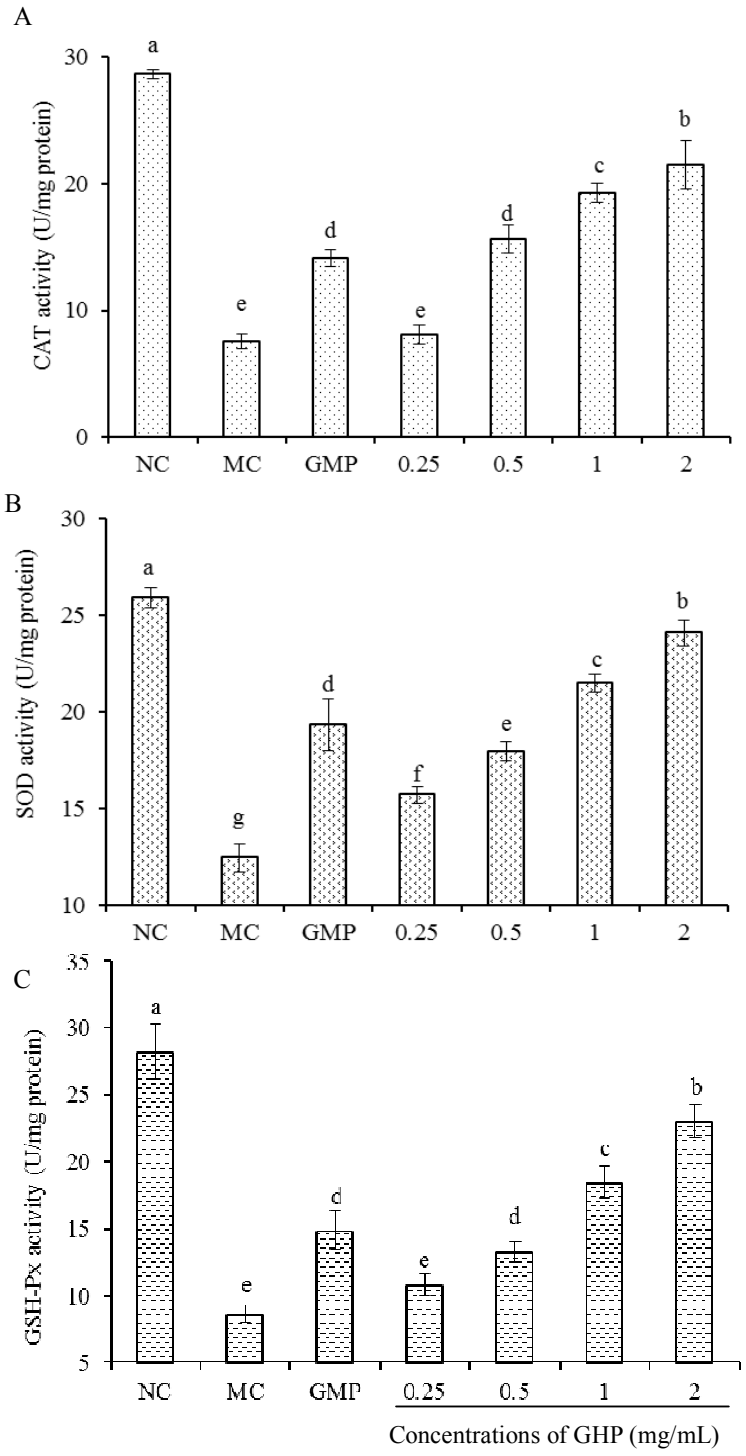


Figure 5

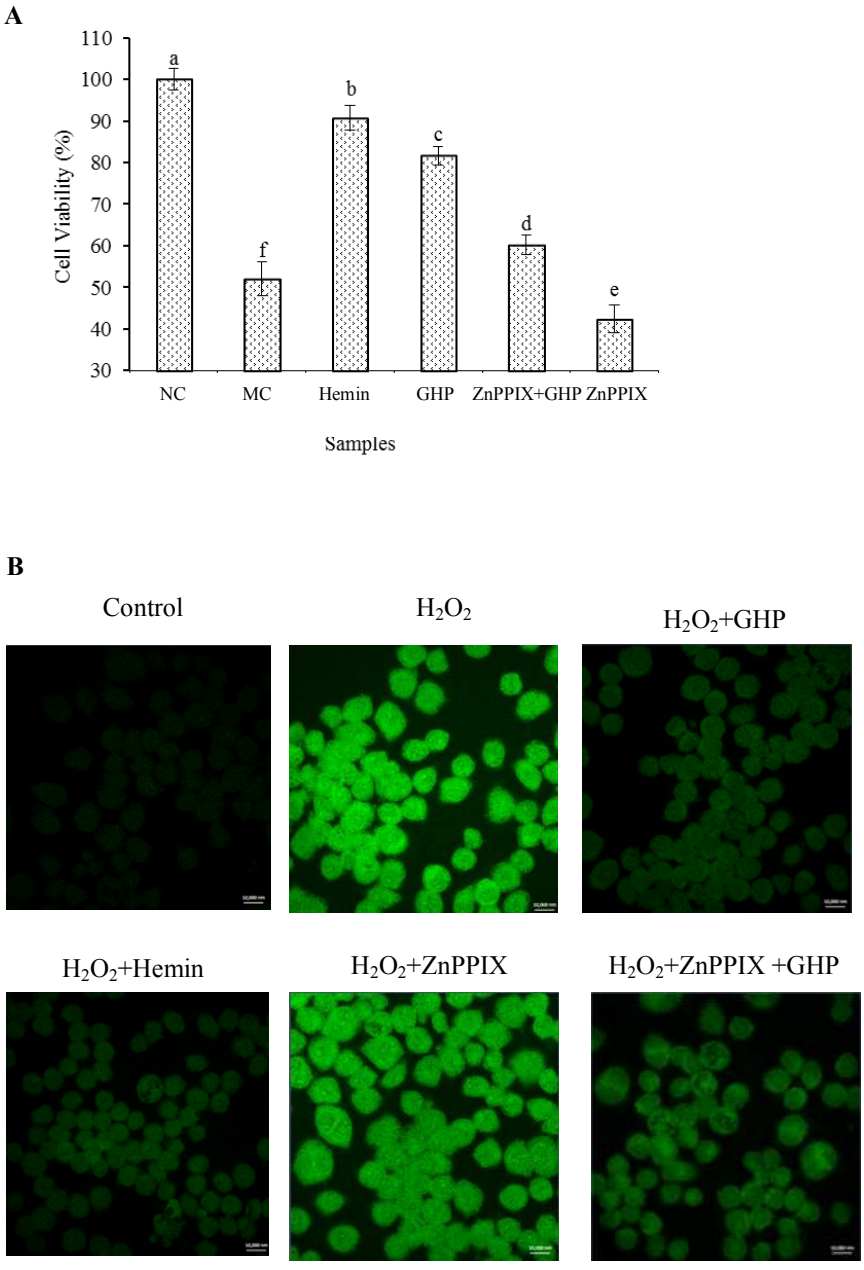


Figure 6

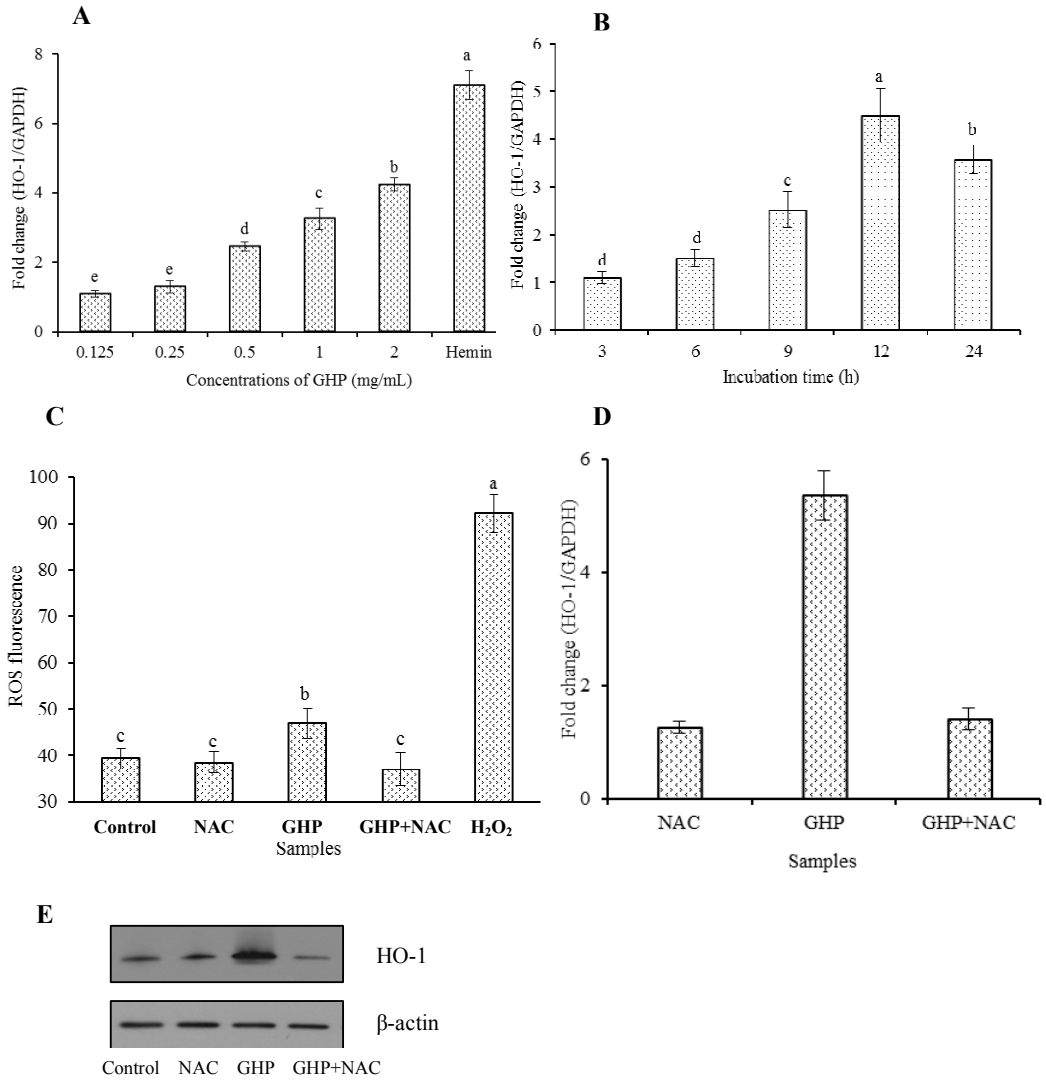


Figure 7

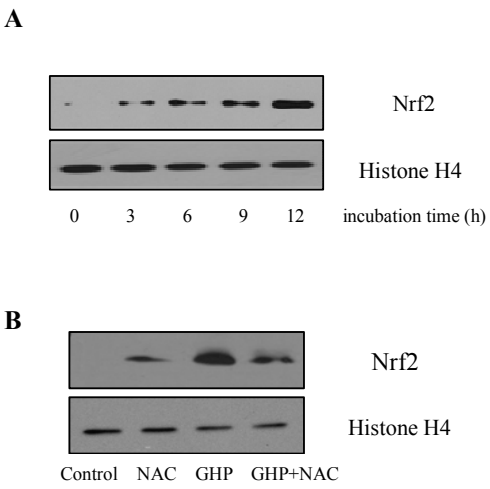
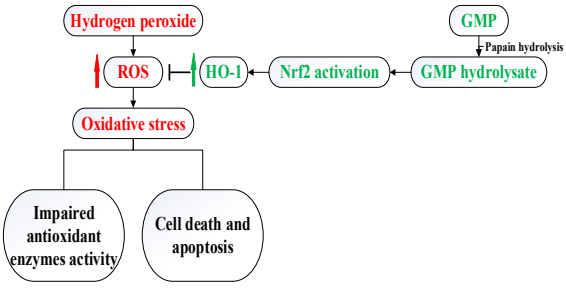


Table 1

Amino acid	Amino acid content of GMP and GHP (μg mg^{-1} protein)	
	GHP	GMP
Asp	73.83	52.2
Thr	125.42	152.37
Ser	59.59	62.05
Glu	169.19	144.29
Gly	12.94	7.39
Ala	79.01	43.82
Val	73.66	69.14
Met	6.04	14.68
Ile	88.80	77.41
Leu	25.44	12.9
Phe	8.54	4.71
Lys	51.25	43.14
His	1.48	2.13
Arg	3.58	4.13
Pro	84.04	91.61



Casein glycomacropeptide hydrolysate had antioxidant activity and exerted protective actions against H_2O_2-induced oxidative stress via induction of Nrf2-mediated HO-1expression in RAW 264.7 macrophages.