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Attenuated effects of peptides derived from porcine plasma albumin on *in vitro* lipid peroxidation in the liver homogenate of mice

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ABSTRACT

Porcine plasma albumin was hydrolyzed with alcalase available for industrial application, and attenuated effects of peptides were evaluated using 4-Nitroquiunoline 1-oxide (4-NQO) as an inducing reagent. 4-NQO is a potent oral carcinogen, which has been found to induce lipid peroxidation *in vivo* and *in vitro*. Our results indicated that addition of 4-NQO resulted in increase of hydroxyl and superoxide radicals, glutathione peroxidase (GPx) activity and levels of malondialdehyde (MDA), and decrease in activities of superoxide dismutase (SOD) and catalase (CAT) and levels of glutathione (GSH). Simultaneous addition of peptides significantly attenuated lipid peroxidation. The lowermost molecular weight (MW) peptide fractions (<3 kDa) had the highest activity. This study also demonstrated that the attenuated effects of peptides might be due to the protective interactions between cells and peptides rather than the direct inhibition of 4-NQO by peptides. The results of this study showed the potential of utilizing porcine plasma albumin as a source of functional peptides.

1. Introduction

Animal blood produced during slaughter is one of the valuable protein sources. There have been many possibilities to use it in food, feed, laboratory, medical, industrial, and fertilizer areas. However, most blood released from slaughterhouses is not efficiently utilized and is mostly discarded without proper disposal treatment, which may cause pollution of water in developing countries (Park & Hyun, 2002). In China, about 1.5 million tons porcine blood is produced every year, containing proteins equal to that of 2.5 million tons of whole eggs, but the amount utilized is very limited. Considering the nutritious and functional properties of the plasma proteins, it is obviously necessary to develop a new method to utilize animal blood.

In recent years, hydrolyzation of proteins with proteolytic enzymes to produce bioactive peptides has become a new biotechnological method for utilizing food processing residues. The functional properties include inhibition of angiotensin I-converting enzyme (ACE), immunomodulatory effects, antimicrobial activity, antithrombotic activity, antioxidant activity, mineral binding properties, etc. (Gill, López-Fandiño, Jorba, & Vulfson, 1996; Meisel, 1997). Among these activities, antioxidant activity has been studied extensively. It is well known that lipid peroxidation occurring in food products causes deterioration in food quality, affecting colour, flavour, texture and nutritional value. In addition, it has been recognized that oxidative stress plays a significant role in a number of diseases. For example, many studies have shown increased oxidative damage to all the major classes of biomolecules in the brains of Alzheimer's patients (Halliwell, 2001; Liu, Raina, Smith, Sayre, & Perry, 2003). Furthermore, cancer is probably a consequence of oxidative DNA damage (Collins, 2005). Lipid peroxidation is inhibited by antioxidative agents. However, since artificial antioxidants pose potential risks *in vivo*, their use in foodstuffs is restricted or prohibited in some countries. Therefore, there is a growing interest in finding safe and natural antioxidants that enhance the body's antioxidant defenses through dietary supplementation (Finkel & Holbrook, 2000).

Animal blood proteins have been investigated also to produce several bioactive peptides, such as ACE inhibitory peptides (Hyun & Shin, 2000), opioid peptides (hemorphins) (Zhao, Garreau, Sannier, & Piot, 1997), and bacterial growth stimulating peptides (Zhao, Coeur, & Piot, 1997). However, it has not been reported on antioxidant activity of porcine plasma albumin peptides. Little attention has been focused on the effects of porcine plasma albumin peptides on lipid peroxidation.

In our previous study, it had been demonstrated that porcine plasma albumin peptides were found to have antioxidative activities (Wang et al., in press). 4-NQO is a potent oral carcinogen and has been found to induce lipid peroxidation *in vivo* and *in vitro*. In this report, the subfractions of the peptides were further investigated to examine their attenuated effects on lipid

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peroxidation induced by 4-NQO. Moreover, the effect of molecular weights of peptides on their functional properties was also evaluated.

2. Materials and methods

2.1. Materials

4-NQO, 1-Chloro 2,4-dinitrobenzene (CDNB), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), GSH and NADPH were purchased from Sigma Chemical Co (St Louis, MO, USA). All chemicals and reagents used were of analytical grade.

2.2. Preparation of active peptide fractions

Methodologies for blood sample preparation and separation of plasma proteins were described in our previous report (Wang et al., in press). Porcine plasma albumin was treated with alcalase (Novo Nordisk, Bagsvaerd, Denmark) to produce peptides. Proteolytic reaction was carried out by adding alcalase at the concentration of 0.1% (w/w). The reaction temperature and pH were adjusted to 55 °C and 7.5, respectively. Samples withdrawn from proteolytic mixture were immediately heated in boiling water for 10 min, followed by centrifugation at 13195 g for 15 min. Peptide concentrations were measured by Lowry method. The separation methods of peptide fractions were also described in our previous report. Active peptide fractions (MW<3 kDa, 3–6 kDa, 6–10 kDa, 10–30 kDa and >30 kDa) were used for this study.

2.3. Preparation of liver homogenate for in vitro experiment

Modified method of Tripathi, Upadhyay, and Chaturvedi (2001) was used to prepare the liver homogenate. Male Sprague–Dawley rats weighing about 200–250 g were sacrificed under ether, which was maintained according to animal/human ethical committee. The liver was excised and washed in ice cold 0.85% saline, weighed, and minced. Five percent (w/v) homogenate was prepared in 50 mM pH 7.4 sodium phosphate buffer and immediately stored at −20 °C. The protein content of the liver tissue was measured by Lowry method. The total protein content was expressed as milligram per gram of tissue.

2.4. Experiment treatments design

This experiment consisted of four different groups. Group A (control) contained 5% liver homogenate only. Group B (4-NQO) consisted of 5% liver homogenate and 0.1 mM 4-NQO. Group C (4-NQO + peptides) had 5% liver homogenate, 0.1 mM 4-NQO and 1 mg/ml peptides. Group D (peptides) contained 5% liver homogenate and 1 mg/ml peptides. These reactions were carried out at 37 °C. Samples were collected at 20 min intervals and used for lipid peroxidation and antioxidant assays.

2.5. HPLC Analysis

The mixture of 0.1 mM 4-NQO and 1 mg/ml peptides was incubated at 37 °C. Samples were collected at 20 min intervals and analyzed using HPLC system. The column was Nova-Pak C18 (3.9 × 150 mm, 4 μm, Waters, State USA) and the mobile phase was constituted of solvent A (water) and solvent B (methanol) with gradient elution, i.e., increasing from 20% to 80% within 25 min and then drastically to 100% within 5 min. The flow rate was 1 ml/min and the detection was made at 355 nm.

2.6. Determination of lipid peroxidation

MDA levels were determined by monitoring TBA reactive substances according to the method of Ohkawa, Ohishi, and Yagi (1979) with minor modifications. The reaction mixture contained 0.2 ml of test sample, 0.2 ml of 8.1% sodium dodecylsulfate (SDS), 1.5 ml of acetic acid and 1.5 ml of 0.5% of TBA. The mixture was heated in a water bath at 95 °C for 60 min. After cooling, 5 ml of *n*-butanol/pyridine (15:1, v/v) was added and shaken well. After centrifugation at 4000 rpm for 10 min, the absorbance of the organic layer was measured at 532 nm in a Shimadzu UV spectrophotometer. 1, 1, 3, 3-tetramethoxypropane was used as standard and the level of lipid peroxidation was expressed as nanomoles of MDA per milligram of tissue protein.

2.7. Scavenging activity of hydroxyl radical

The deoxyribose assay was used to determine the hydroxyl radical scavenging activity (Halliwell, Gutteridge, & Aruoma, 1987). 0.2 ml of test sample was added to the reaction mixture containing 100 μM $FeCl_3$, 100 μM EDTA, 1 mM H_2O_2 and 2.8 mM 2-deoxy-D-ribose. The volume was made up to 1 ml with 0.02 M pH 7.4 phosphate buffer and incubated at 37 °C for 1 h. The mixture was then heated to 95 °C in a water bath for 15 min, followed by the addition of 1 ml each of 2.8% of trichloroacetic acid (TCA) and 0.5% of TBA. After cooling, it was centrifuged at 4800 rpm for 15 min. The absorbance of the supernatant was measured at 532 nm. The percentage hydroxyl radical scavenging activity was determined accordingly in comparison with the negative control.

2.8. Scavenging activity of superoxide radical

Superoxide radical scavenging activity was measured according to the method of Lee, Kim, Kim, and Jang (2002) with minor modifications. Samples were added to the reaction mixture containing 100 μl of 0.03 M ethylenediaminetetraacetic acid (EDTA), 10 μl of 0.03 M hypoxanthine in 0.05 M NaOH, and 200 μl of 1.42 mM nitroblue tetrazolium. The volume was made up to 2.9 ml with 0.02 M pH 7.4 phosphate buffer. After the solution was pre-incubated at ambient temperature for 3 min, 100 μl of 0.5 U/ml of xanthine oxidase solution was added to the mixture. The solution was incubated at ambient temperature for 20 min and the absorbance of the solutions was measured at 560 nm. The percentage superoxide radical scavenging activity was determined accordingly in comparison with the negative control.

2.9. Antioxidant enzyme activities

2.9.1. Determination of superoxide dismutase

The activity of SOD was measured according to the method of Carrillo, Kanai, Sato, Ivy, and Kenichi (1992) with modifications. The enzyme was detected based on its ability to inhibit the superoxide-mediated reduction of cytochrome *c* by xanthine oxidase and xanthine. One hundred microliter of the sample was added to 2 ml of 0.05 M pH 7.4 phosphate buffer, which contained 100 μM EDTA, 100 μM xanthine, 40 μM cytochrome *c* and 0.01 U xanthine oxidase. The reaction was started by adding xanthine oxidase and then conducted at 30 °C for 3 min. The absorbance was measured at 550 nm in a Shimadzu UV spectrophotometer. One enzyme unit was defined as the amount of enzyme required to inhibit the cytochrome *c* reduction by 50%. The enzyme activity was expressed as units per milligram of tissue protein.

2.9.2. Determination of catalase

The activity of CAT was assayed according to the method of Carrillo et al. (1992) with minor modifications. The mixture of

0.05 M pH 7.4 sodium phosphate buffer, 1 μM H_2O_2 and the sample was made up a final volume of 3 ml, and the decrease in absorbency was measured at 240 nm in 1 min. One unit of CAT activity was defined as the amount of enzyme required to decompose 1 μM of H_2O_2 in 1 min. The enzyme activity was expressed as units per gram of tissue protein.

2.9.3. Determination of glutathione peroxidase

GPx activity was determined by the method of Wendel (1981) with modifications. The mixture contained 0.6 ml of 0.25 M pH 7.0 phosphate buffer, 0.3 ml of 10 mM GSH, 0.3 ml of 10 mM EDTA, 0.3 ml of 10 mM sodium azide, 0.3 ml of 2 mM NADPH and 20 μl glutathione reductase. The mixture was added to 0.9 ml of the sample and incubated at 30 °C for 5 min. The reaction was initiated by the addition of 0.3 ml of 2.5 mM hydrogen peroxide. The absorbance was immediately read at 340 nm. The enzymatic activity was expressed as nanomole NADPH oxidized per minute per milligram of protein. The amount of NADPH within the samples was determined by using a standard curve with known amounts of NADPH.

2.10. Determination of reduced glutathione

Reduced glutathione was determined by the method of Moron, Depierre, and Mannervik (1979). One milliliter of test sample was precipitated by 10% TCA, and centrifuged. The supernatant was added 4 ml of 0.2 M phosphate buffer (pH 8.0), and 0.5 ml of 0.6 mM DTNB, and incubated for 10 min at room temperature. The absorbance was measured at 412 nm. To make the standard curve, 10 mg standard substance of GSH was dissolved in 100 ml of distilled water. The GSH concentration was calculated from the standard curve. The amounts of glutathione in tissues were expressed as milligram per gram of tissue protein.

2.11. Statistical analysis

Experimental data were expressed as mean ± standard deviation (SD). Data analysis was carried out using SPSS software, version 12.0. Differences among groups were evaluated by one-way analysis of variance. When significant differences were indicated by analysis of variance, group means were compared using the Duncan test. A probability of $P < 0.05$ was considered as significant.

3. Results

3.1. HPLC Analysis

Fig. 1 shows the HPLC results of 4-NQO and peptides in incubation time. It was found that retention time and peak area of 4-NQO did not change significantly with increase in time.

3.2. Lipid peroxidation

Lipid peroxidation was evidenced by the formation of MDA. Fig. 2 depicts the levels of MDA in different groups. In group A, there was no significant change in the production of MDA with increase in time. Similar result was observed in group D. However, as time advanced, there was a significant ($p < 0.01$) increase in the levels of MDA in group B, which also showed the highest level of MDA among all groups. Compared to group B, simultaneous addition of peptides (group C) had a significant ($p < 0.05$) inhibition in the levels of MDA at all times of incubation. Within group C, the ability of MW <3 kDa peptides to inhibit lipid peroxidation was significantly ($p < 0.05$) higher than that of other MW peptides.

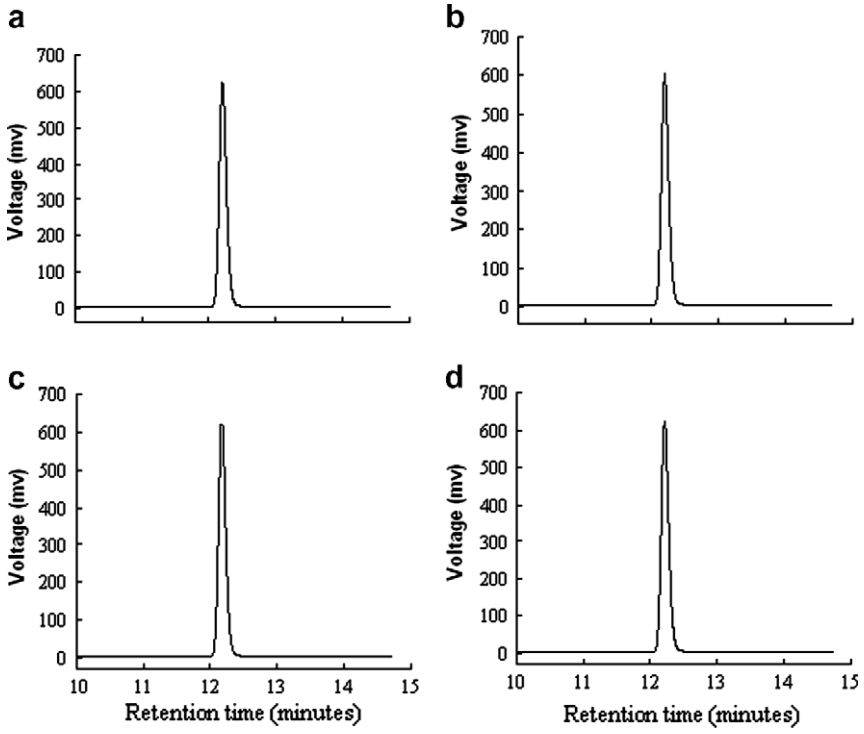

Fig. 1. HPLC profiles of the incubation mixture. The samples were determined at different times: (a) 0 min; (b) 20 min; (c) 40 min; (d) 60 min at 355 nm separately. By Hao Zhang, Jinzhi Wang, Ran Li, Jing Bai, Yubin Ye, Fazheng Ren*.

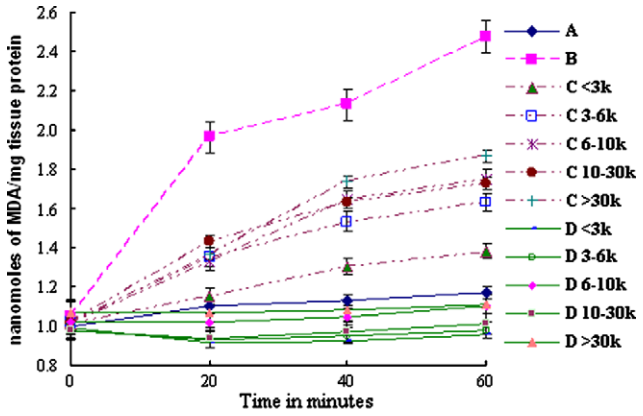

Fig. 2. Levels of MDA in different groups. Experimental data are expressed as mean ± SD. By Hao Zhang, Jinzhi Wang, Ran Li, Jing Bai, Yubin Ye, Fazheng Ren*.

3.3. Scavenging activity of hydroxyl and superoxide radicals

Fig. 3 depicts the scavenging activity of hydroxyl and superoxide radicals in different groups. In the control group A, there was no significant change in the scavenging activity of hydroxyl and superoxide radicals as time advanced. Compared to group A, there was a significant ($p < 0.05$) increase in scavenging activity in group D. On the other hand, a significant ($p < 0.01$) decrease was observed in the scavenging activity in group B in a time dependent manner, which was significantly ($p < 0.01$) lower than other groups. In group C the scavenging activity of MW <3 kDa peptides was significantly ($p < 0.05$) higher than that of other peptides.

3.4. Antioxidant enzyme activities

The activities of SOD and CAT are presented in Fig. 4. In group A, there was no significant change in the activities of SOD and CAT as time advanced. Compared to group A, it was also observed in group D that there was no significant increase in the activities of SOD and CAT. However, a significant ($p < 0.01$) decrease was observed in the activities of SOD and CAT in group B as compared with control. Compared to group B, there was a significant ($p < 0.05$) inhibition of the decrease of SOD and CAT activities in group C as time advanced. The activity of CAT with addition of MW <3 kDa peptides was significantly higher than that of other peptides at 60 min.

GPx activity is shown in Fig. 5. As time advanced, GPx activity was no significant change in group A. Compared to group A, it was also observed in group D that there was no significant ($p > 0.05$) decrease in GPx activity. There was a significant ($p < 0.01$) increase in GPx activity in group B. Compared to group B, there was a significantly ($p < 0.05$) inhibit the increase of GPx activity in group C at all times of incubation.

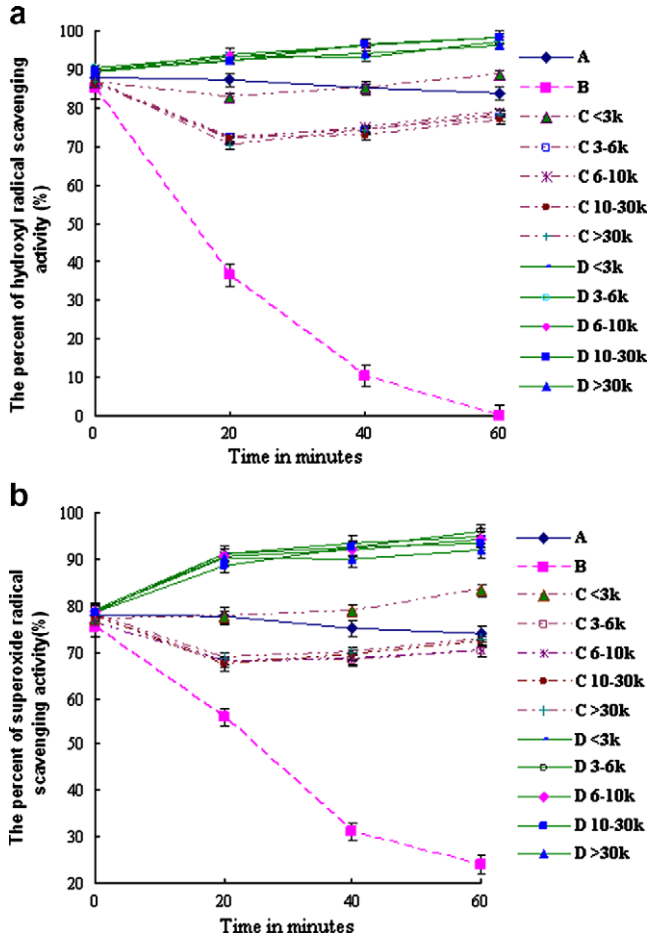

Fig. 3. The percent of hydroxyl and superoxide radicals scavenging activity in different groups. Experimental data are expressed as mean ± SD. (a) The percent of hydroxyl radical scavenging activity in different groups. (b) The percent of superoxide radical scavenging activity in different groups. By Hao Zhang, Jinzhi Wang, Ran Li, Jing Bai, Yubin Ye, Fazheng Ren*.

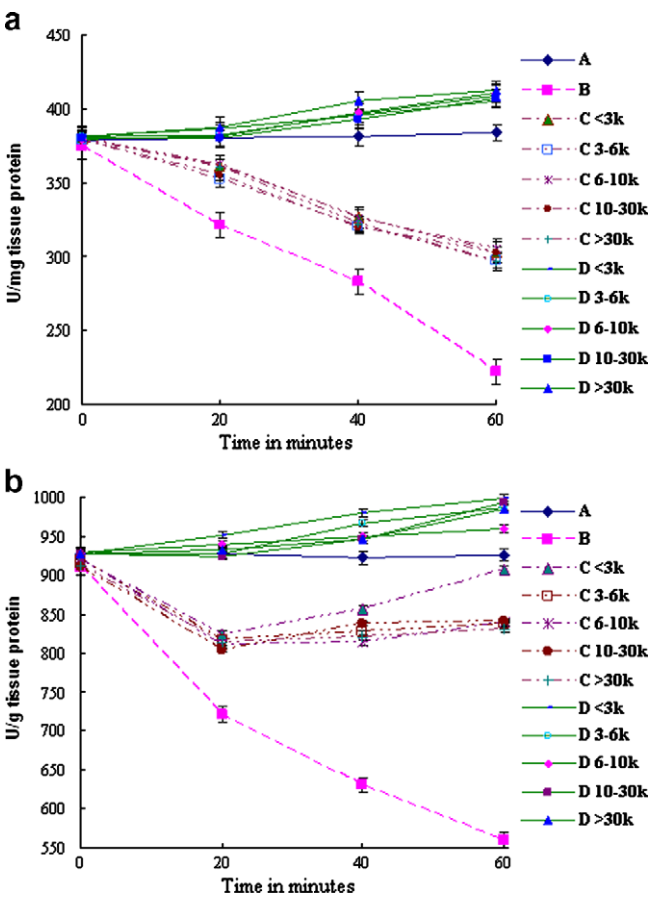

Fig. 4. Activities of SOD and CAT in different groups. Experimental data are expressed as mean ± SD. (a) Activity of SOD in different groups. (b) Activity of CAT in different groups. By Hao Zhang, Jinzhi Wang, Ran Li, Jing Bai, Yubin Ye, Fazheng Ren*.

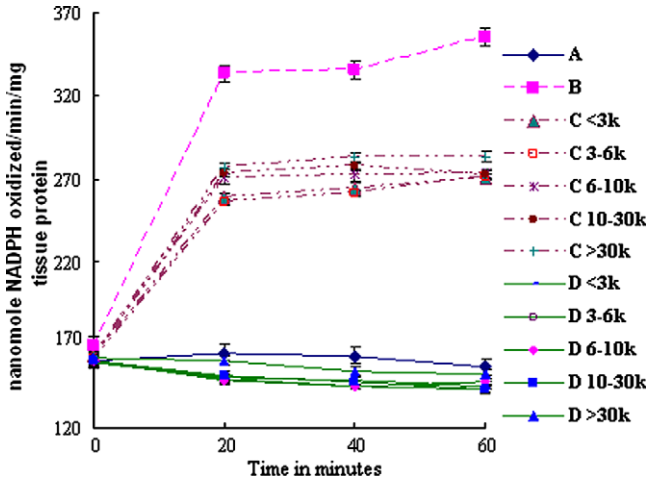

Fig. 5. Activity of GPx in different groups. Experimental data are expressed as mean ± SD. By Hao Zhang, Jinzhi Wang, Ran Li, Jing Bai, Yubin Ye, Fazheng Ren*.

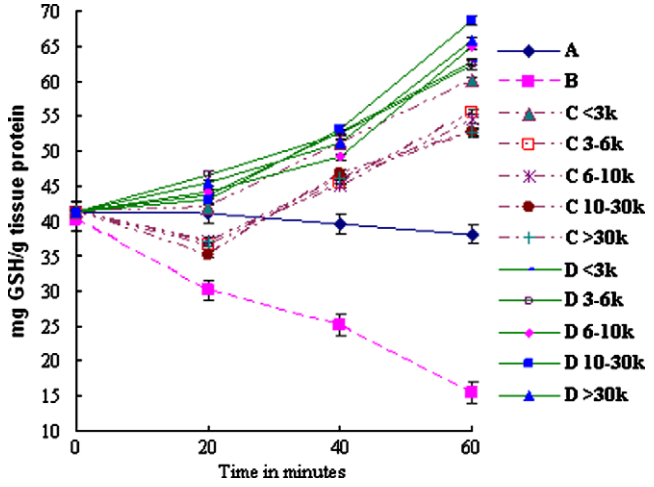

Fig. 6. Levels of GSH in different groups. Experimental data are expressed as mean ± SD. By Hao Zhang, Jinzhi Wang, Ran Li, Jing Bai, Yubin Ye, Fazheng Ren*.

3.5. Levels of glutathione

The levels of GSH are shown in Fig. 6. In the control group, along with time there was no significant ($p > 0.05$) decrease in the levels of GSH. Group B, underwent a significant ($p < 0.05$) decrease in the levels of GSH with increase in time as compared with control. On the other hand, the levels of GSH were found to be significant ($p < 0.05$) increased in group C and D as time advanced. In addition, in group C, the levels of GSH with addition of MW <3 kDa peptides were markedly ($p < 0.05$) higher than those of other MW peptides at all incubation times.

4. Discussion

In this study, MDA was used as a marker of the levels of lipid peroxidation. Our data demonstrated that porcine plasma albumin peptides could inhibit the lipid peroxidation induced by 4-NQO *in vitro*. If peptides react with 4-NQO and change its structure, 4-NQO can no longer undergo redox cycling and induce lipid peroxidation. In our present study, the HPLC method was used to examine whether peptides react with 4-NQO. It was found that retention time and peak area of 4-NQO did not change significantly with time (Fig. 1). The results showed that the peptides did not react with 4-NQO during incubation. Therefore, it was speculated that the attenuated effects of peptides might be due to the protective interactions between cells and peptides rather than the direct inhibition of 4-NQO by peptides.

4-NQO has been shown to induce a potent intracellular oxidative stress (Nunoshiba & Demple, 1993). It can undergo redox cycling and produce free radicals (Ramotar, Belanger, Brodeur, Masson, & Drobetsky, 1998). These free radicals include superoxide radical and hydroxyl radical, which induce lipid peroxidation by chain oxidation (Yamamoto, Takahashi, & Niki, 1987). In this work, the peptides were shown to be good free radical scavengers (Fig. 3) and the addition of peptides (group C) was shown great activity of inhibiting lipid peroxidation. Therefore, scavenging of the free radicals induced by 4-NQO was a possible reason for peptides to inhibit lipid peroxidation. In addition, it was found that the lowermost molecular weight peptide fractions (MW <3 kDa) had the highest activity. These results are in line with observations of other studies (Li, Chen, Wang, Ji, & Wu, 2007; Pihlanto-Leppälä, 2001) and strengthen the fact that functional properties of antioxidant peptides are highly influenced by their molecular structure and molecular weight (Suetsuna, Ukeda, & Ochi, 2000). With lower molecular weights, these peptides have higher chance to cross the cell membrane and exert a biological effect (Roberts, Burney, Black, & Zaloga, 1999).

Furthermore, the results showed that peptides protected antioxidant system of cells from the damage induced by 4-NQO. It is well known that, oxidative metabolism is essential for the survival of cells with the production of free radicals that cause oxidation. Therefore, cells have cooperative defense systems for the reduction of oxidation. The defense systems contain numerous enzymatic and nonenzymatic antioxidants, including SOD, CAT, GPx and GSH (Meister, 1994). However, when excess oxidations occur, they can overwhelm protective systems.

CAT catalyzes the conversion of hydrogen peroxide to water and molecular oxygen. Another important key enzyme involved in the dismutation of superoxide radical is SOD. It is commonly accepted that SOD protects against the free radical injury by converting superoxide radical to H_2O_2 and prevents the formation of hydroxyl radical. In group B there was a significant decrease in the activities of SOD and CAT (Fig. 4). Srinivasan, Sabitha, and Shyamaladevi (2007) also reported similar results. Excess free radicals have been shown to react with several amino acid residues *in vitro*, making active enzymes denatured (Stadtman & Berlett, 1998). The increased production of free radicals in a time dependent manner competes and inactivates the antioxidant enzymes (Srinivasan et al., 2007). The simultaneous addition of peptides (group C) had a significant reduction in free radical production (Fig. 3). As a result, there was a significant ($p < 0.05$) inhibition of the decrease of SOD and CAT activities in group C (Fig. 4).

GPx also protects the cell protein and membrane against oxidation. GPx reduces variable hydroperoxides at the expense of glutathione and/or other reducing equivalents (Brigelius-Flohe, 1999). Therefore, the depletion in GSH contents after exposure to 4-NQO or the production of free radicals may evoke the activity of GPx. GSH acts as a cofactor with the enzyme GPx to detoxify hydrogen peroxide and lipid peroxides in cells and tissues. GSH is a multifunctional tripeptide present in most living cells at relatively high concentrations with functions of controlling many cell functions and protecting cells against xenobiotic and free radical induced toxicity. In the present *in vitro* study, the depletion in GSH contents after exposure to 4-NQO may be due to the reaction of GSH with free radicals, resulting in the formation of thiyl radicals that react to produce oxidized glutathione (GSSG) (Navarro et al., 1997). GSH serves as a substrate for GPx, so the enhanced activity of GPx in group B may be another reason for the reduction

of GSH levels. On the other hand, in group C peptides significantly reversed the reduction of GSH levels induced by 4-NQO (Fig. 6). This effect indicated that peptides could offer protection against 4-NQO induced decrease in GSH levels. It was also observed in group D that GSH levels were significantly higher than in the control group. This observation may be due to the reducing power of peptides, which reduced GSSG to GSH. The protected antioxidant system could cooperate with peptides to inhibit lipid peroxidation efficiently.

It was concluded from these results that the activity of peptides was not a direct chemical inhibition of 4-NQO. Scavenging of the free radicals and protection of antioxidant system might be the major mechanisms of their attenuated effects on lipid peroxidation. Low molecular weight peptide fractions (MW <3 kDa) had the highest activity of inhibiting lipid peroxidation. These suggested that antioxidant peptide fractions from porcine plasma albumin might be useful for food additives, diet nutrients and pharmaceutical agents. However, further studies are needed to investigate the *in vivo* activity of the peptide fractions (MW <3 kDa), the structure of active peptides and their industrial applications.

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