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Food Chemistry 98 (2006) 149–157
Food Chemistry
www.elsevier.com/locate/foodchem

Protective effects of an aqueous extract of Welsh onion green leaves on oxidative damage of reactive oxygen and nitrogen species

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Received 25 October 2004; received in revised form 20 May 2005; accepted 20 May 2005

Abstract

Antioxidant effects of Welsh onion green leaves on reactive oxygen and nitrogen species were investigated. The results showed that aqueous extract of Welsh onion green leaves (WOE) in the range 0.05–1.0 mg/ml showed a potent concentration-dependent reduction of xanthine oxidase activity, and this inhibitory action of WOE correlated well with total flavonoid content ($r = 0.99$, $p < 0.05$). Furthermore, WOE also showed scavenging of superoxide radical ($p < 0.05$), hydroxyl radical ($p < 0.05$), nitric oxide ($p < 0.05$) and chelating metal ions ($p < 0.05$) in a dose-dependent manner. In addition, the oxidative damage of albumin, induced by hydroxyl radical (·OH) and hypochlorous acid (HOCl) in an acellular system, was inhibited by 0.1–2.0 mg/ml of WOE. Protein tyrosine residue nitration in mouse heart homogenates was obviously decreased by 2 mg/ml WOE. Thus, these positive effects might contribute to the protective effect of Welsh onion against oxidative damage of protein.

Keywords: Welsh onion; Antioxidant; Reactive oxygen species; Nitric oxide; Protein oxidation

1. Introduction

A series of reports suggested that diets rich in fruit and vegetables have some therapeutic effects on anti-inflammatory (Rossi et al., 2003), anti-hepatotoxic (Hattori, Yamada, Nishikawa, Fukuda, & Fujino, 2001; Lee, Campbell, Molyneux, Hasegawa, & Lee, 2001), and anti-carcinogenic activities (Vang, Rasmussen, & Andersen, 1997). These protective effects have been attributed to certain antioxidants,including plasma retinal and alpha-tocopherol with strong inverse associations between levels of DNA adducts (Palli et al., 2003), alpha-carotene with anti-carcinogenic activities (Nishino, 1998) and anthocyanins with protective effects against acute lung inflammation (Rossi et al., 2003). These chemoprotective reactions were due to scavenging of free radicals (Aldini, Carini, Piccoli, Rossoni, & Facino, 2003), chelating of trace metal elements (e.g., iron and copper) (Borsari et al., 2001) or inhibition of some enzymes involved in formation of free radicals (e.g., xanthine oxidase, lipooxygenase, NADPH oxidase and cyclooxygenase) (Lampe, 1999). These reports suggest that the inhibition of enzymes or scavenging activities against free radicals are responsible for the protective power in evaluating diets. Therefore, increasing ingestion of such diets may help in maintaining good health. However, under physiological conditions, there is a continuous production of free radicals, such as

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doi:10.1016/j.foodchem.2005.05.057

superoxide radicals, hydroxyl radicals and nitric oxide (Cuzzocrea, Riley, Caputi, & Salvemini, 2001). These reactive species are essential for life in lower quantities, because they are involved in cell signalling and physiological processes. In addition to these necessary functions, non-essential reactive oxygen species (ROS) or reactive nitrogen species (RNS) react with cell molecules, induce protein oxidation and are responsible for harmful damage in cells and tissues where there are generated. For example, neutrophils and macrophages could generate mass superoxide or NO in response to different extracellular stimulants (e.g., lipopolysaccharide and cytokines) (Jersmann, Rathjen, & Ferrante, 1998). In addition to their microbicidal activity, excessive production of ROS or RNS has been suggested to accelerate a number of pathological processes, such as cardiovascular diseases (Sohn et al., 2003), liver failure (Wheeler et al., 2001) and cancer (Byun, Henderson, Mueller, & Heinecke, 1999).

Recent studies, in different experimental models, have considered the potential of different dietary antioxidants to help prevent development of oxidative damage (Borek, 2001; Rao, 2002). Welsh onion (*Allium fistulosum* L., Alliaceae), an important flavouring vegetable in Asian dishes and commonly called Ching-Tsung, has been reported to inhibit LDL oxidation (Wang, Chen, Liang, & Duh, 2005), modulate aortic vascular tone (Chen, Tsai, & Chen, 1999) and lower blood pressure (Chen, Chen, Tsai, & Jen, 2000). Other members of the *Allium* family (e.g., garlic and onion) have been used to treat a variety of diseases, including hypertension and atherosclerosis in many countries (Hasler, Kundrat, & Wool, 2000; Rahman, 2001). *Allium* vegetables have been proved to raise antioxidant capacity in vitro and in vivo (Borek, 2001). For example, garlic and different garlic extracts have beneficial effects on blood lipid and low-density lipoprotein oxidation. Many studies have suggested that these beneficial effects are mainly due to organosulfur compounds. Protective effects of organosulfur compounds (e.g., *S*-allyl cysteine and diallyl disulfide) derived from garlic have been found in scavenging ROS (Prasad, Laxdal, Yu, & Raney, 1996), inhibiting low-density lipoprotein oxidation (Ou, Tsao, Lin, & Yin, 2003) and suppressing the formation of atherogenic lesions (Ho, Ide, & Lau, 2001). There is a positive correlation between related aging diseases and protein oxidation in tissue. Protein oxidation results in protein denaturation and loss of their function, which is regarded as a marker of tissue damage and aging. Consequently, inflammatory processes, atherosclerosis and cancer are concomitant with the development of protein oxidation (Cuzzocrea et al., 2001). Although, previously, Welsh onion has been shown to inhibit low-density lipoprotein oxidation in vitro, whether Welsh onion could directly scavenge radicals and protect protein from oxidative damage has not been clearly elucidated. Thus, the scavenging effects of Welsh onion on reactive oxygen and nitrogen species were investigated. The anti-protein oxidation by Welsh onion was also evaluated.

2. Materials and methods

2.1. Materials

Briefly, fresh mature Welsh onions were obtained from ten local markets in Tainan, Taiwan. These batches of onion were mixed well, and then used for WOE preparation. (2-Aminoethyl) diphenyl borate, xanthine oxidase, hypoxanthine, NADH, phenazine methosulphate, nitroblue tetrazolium, deoxyribose, thiobarbituric acid and sodium nitroprusside were purchased from Sigma (St. Louis, MO, USA). Ferrozine, trolox and dinitrophenylhydrazine were purchased from Aldrich (St. Louis, MO, USA).

2.2. Preparation of aqueous extract from Welsh onion green leaves juice

The green portion (1000 g) of Welsh onion was squeezed and filtered by a food processor (National, Model MJ-C85; Tokyo, Japan; pore size of the filter: 0.2 mm), ten times, to obtain the Welsh onion green leave juice (400 g). After centrifugation at 10,000*g* for 30 min, the supernatant (340 g) was the aqueous extract of Welsh onion green leave (WOE). Furthermore, the extract was filtered, frozen at −80 °C, and then lyophilized for 24 h. This dehydrated power (20 g) was then dissolved in phosphate buffer saline for each assay and used for the determination of total flavonoid content.

2.3. Assay of total flavonoid content

One ml of WOE (0.05–1.0 mg/ml) was incubated with 0.1 ml (2-aminoethyl) diphenyl borate (0.2% in ethanol). The absorbance was measured at 405 nm after 20 min of incubation (Oomah & Mazza, 1996). The absorbance of rutin solutions was detected under the same conditions. The amount of flavonoids in WOE (in rutin equivalents) was calculated.

2.4. Assay of inhibitory activity against xanthine oxidase

The xanthine oxidase activity was measured spectrophotometrically by detecting uric acid formation at 295 nm. Uric acid is generated from enzymatic oxidation of hypoxanthine by xanthine oxidase (Chang, Chang, Lu, & Chiang, 1994). The reaction were started by addition of various concentrations of WOE, 1 unit/ml of xanthine oxidase and 0.4 mM hypoxanthine in 0.05 M KH_2PO_4 buffer (pH 7.4) and incubated at 37 °C for 15 min.

2.5. Assay of scavenging activity against superoxide

Superoxide radical was generated from a NADH–phenazine methosulphate (PMS) system and quantified by measurement of the reduction of nitroblue tetrazolium (NBT) (Rao, 1989). Briefly, various concentration of WOE were incubated with 0.1 ml of PMS (0.1 mM), 0.1 ml of NBT (1 mM) and finally make up to 0.9 ml with 0.05 M KH_2PO_4 buffer (pH 7.4). The reaction mixtures were initiated by addition of 0.1 ml of NADH (2 mM). After incubation at 25 °C for 10 min, the absorption at 570 nm was read. Trolox was used as positive control.

2.6. Assay of scavenging activity against hydroxyl radical

Hydroxyl radical, generated from the Fe^{3+}–ascorbate–H_2O_2 (Fenton reaction), was evaluated by degradation of deoxyribose that produced thiobarbituric acid reactive substance (TBARS) (Halliwell, Gutteridge, & Aruoma, 1987). The reaction mixture contained 5 mM deoxyribose, 50 μM $FeCl_3$, 0.1 mM ascorbic acid, 0.1 mM H_2O_2 in 0.02 M KH_2PO_4 buffer (pH 7.4) and various concentrations of the WOE. The reaction mixture was incubated at 37 °C for 30 min. Then, one ml of 1% thiobarbituric acid and 1 ml of 3% trichloroacetic acid were added and heated at 90 °C for 20 min. The TBARS was measured, as previously described, by measuring absorbance at 532 nm. The results were expressed as percentage inhibition of deoxyribose oxidation, as determined by the following formula:

$$\%\text{Inhibition} = [(B - W)/B] \times 100,$$

where B was the malondialdehyde produced by Fenton reaction treatment alone, and W was the malondialdehyde produced in the presence of WOE or trolox.

2.7. Assay of scavenging activity against nitric oxide

Sodium nitroprusside, at physiological pH, spontaneously produce nitric oxide, which interacts with oxygen to generate nitrite (Marcocci, Maguire, Droy-Lefaix, & Packer, 1994). 0.005 M Sodium nitroprusside and various concentration of WOE in PBS (pH 7.4) were incubated at 25 °C for 150 min. After incubation, nitrite produced from sodium nitroprusside was measured by the Griess reaction (1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in water); 0.1 ml of each supernatant was mixed with the same volume of Griess reagent and absorbance of the mixture at 550 nm was determined.

2.8. Assay of chelating activity on metal ion

The chelating activity of WOE on Fe^{2+} was measured. Various concentrations of WOE and 0.1 mM $FeCl_2$ were incubated at 25 °C for 10 min. The mixture was treated with 0.1 mM ferrozine and absorbance determined at 562 nm. EDTA was used as positive control (Carter, 1971).

2.9. Assay of oxidative modification of protein

2.9.1. Protein oxidized by $^{\cdot}OH$

Potassium phosphate buffer (20 mM, pH 7.4) contained 4 mg/ml bovine serum albumin (BSA).BSA was oxidised with 0.05 mM $FeCl_3$, 0.1 mM ascorbic acid, and 1 mM H_2O_2 with phosphate buffer reaction mixture and then samples were incubated for 30 min at 25 °C. WOE and trolox were added to the mixture in the presence of H_2O_2 or not.

2.9.2. Protein oxidized by HOCl

Potassium phosphate buffer (20 mM, pH 7.4) contained BSA. Oxidation of BSA with hypochlorite/hypochlorous acid was carried out by addition of 0.4 mM HOCl (determined using $\varepsilon_{290} = 350\ M^{-1}\ cm^{-1}$) to BSA with phosphate buffer, as previously described. WOE and trolox were added to mixture in the presence of HOCl or not.

2.9.3. Assay of protein oxidation

Effects of WOE on protein oxidation were detected, as previously reported (Lenz, Costabel, Shaltiel, & Levine, 1989). These protein oxidations were evaluated by determining the number of carbonyl groups on proteins using 2,4-dinitrophenylhydrazine (DNPH), as previously reported. Briefly, native BSA and oxidized BSA were treated with 0.05% DNPH at room temperature for 30 min. Following addition of 5% trichloroacetic acid, these samples were stood on ice for 15 min and centrifuged at 5000*g* for 10 min. The protein pellets were washed three times with ethanol–ethyl acetate (1:1, v/v) and dissolved in 6 M guanidine–HCl (pH 2.3). The absorbance of the sample at 370 nm was used to analyze protein carbonyl groups.

2.10. Assay of protein tyrosine residue nitration in mouse heart homogenate in vitro

Hearts of male mice were weighed, minced and the tissue homogenized in five volumes of ice-cold homogenization buffer (0.25 M sucrose, 0.05 M Tris–HCl, pH 7.4, 0.001 M EDTA) at 4 °C. The homogenates were then centrifuged at 1000*g* for 20 min. Then, aliquots of 100 μl of 5 mg/ml heart homogenate supernatants were incubated, with or without WOE and trolox, in the presence of 500 units/ml of horseradish peroxidase (HRP), 0.6 mM sodium nitrite and 0.6 mM HOCl at 37 °C for 6 h. Reactions were stopped by addition of SDS sample buffer and heated at 90 °C for 10 min (Sampson, Ye, Rosen, & Beckman, 1998). Each sample,

which contained 0.05 mg protein, was separated on 8% SDS–polyacrylamide minigels. After electrophoresis, gels were transferred to nitrocellulose paper. The loading and transfer of equal amounts of protein in each lane was verified by staining of the protein bands with Ponceau S. solution (Sigma, USA). After extensive washing with distilled water to remove the protein stain, the membrane was incubated with 5% BSA in phosphate-buffered saline (containing 0.1% Tween-20) to block non-specific immunoglobulins and then immunoblotted, as described with rabbit polyclonal anti-nitrotyrosine (Cayman Chemical, Michigan, USA) or anti-tubulin (Upstate Biotechnology, USA) antibody. Blots were then incubated with a secondary monoclonal peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology, USA). Then, proteins were detected with an enhanced chemiluminescence (ECL) kit (Amersham, USA).

2.11. Statistical analysis

All data were recorded as means ± SD (standard deviation). The StatView statistical package (SAS Institute Inc.) was used to analyze data and statistical significance between the groups was assessed by using one-way analysis of variance (ANOVA), followed by pairwise comparison with a post hoc test with Bonferroni correction. The level of significance was chosen as $p < 0.05$.

3. Results

3.1. Inhibitory effect on xanthine oxidase and total flavonoid content

Fig. 1 shows the inhibitory effects of WOEs on xanthine oxidase activity and their total flavonoid contents. In the range 0.05–1.0 mg/ml, the inhibitory activity on xanthine oxidase was 1.0–97.0% and total flavonoid content with 0.0007–0.0151 mg/ml. The equation of total flavonoid content (Y) and amount of WOE (X) is $Y = 15.8X + 0.11$ ($r = 0.99$, $p < 0.05$). Furthermore, the inhibitory activity on xanthine oxidase by WOE depended on its concentration and correlated well ($r = 0.99$, $p < 0.05$) with its flavonoid content.

3.2. Scavenging of superoxide radicals and hydroxyl radicals

The superoxide scavenging activity was assayed by the NADH–PMS–NBT system (Table 1). The result showed that scavenging of superoxide activity increased with increasing concentration of WOE and trolox. In the range 0.05–1.0 mg/ml, WOE and trolox showed

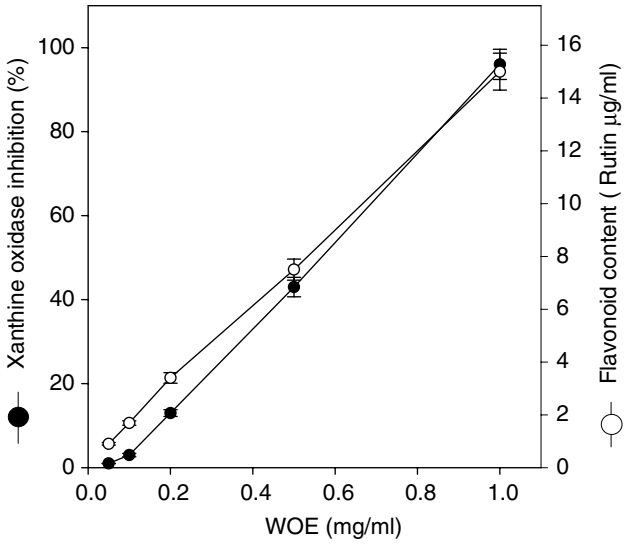

Fig. 1. Inhibitory effect on xanthine oxidase and total flavonoid content of aqueous extracts of Welsh onion green leaves (WOE). The xanthine oxidase activity was measured by detecting uric acid formation. The total flavonoid content was determined as rutin equivalents as described in Section 2. The data are displayed as means ± SD ($n = 5$). Results were analyzed by ANOVA ($p < 0.05$).

scavenging activities against superoxide of 5.0–80.0% and 14.0–87.0%, in the NADH–PMS–NBT model system, respectively. Table 1 also shows the hydroxyl radical scavenging activity of WOE. The hydroxyl radicals generated from the Fe^{3+}–ascorbate–H_2O_2 system caused deoxyribose degradation to TBARS. In the range 0.1–0.5 mg/ml of WOE, TBARS production was decreased by increasing concentration of WOE. The scavenging activity of WOE against hydroxyl radical, in the range 0.05–0.2 mg/ml, was inferior to trolox.

3.3. Chelating of metal ion and scavenging of nitric oxide

The chelating activity of WOE was evaluated by the ferrozine assay, and the results are shown in Fig. 2. The chelating effects of WOE on ferrous ions increased with increasing concentrations. One mg/ml of WOE exhibited 80% chelating effects on ferrous ions. The chelating effect of EDTA, as a positive chelator, at 0.1–1 mg/ml, clearly reached a plateau of 100%. On the other hand, the inhibitory activity against nitric oxide by WOE in the sodium nitroprusside (SNP) system is shown in Fig. 3. SNP ($Na_2[Fe(CN)_5NO]$), a known vasodilator, undergoes one-electron reduction to produce cyanide and NO. At 0.01–1 mg/ml, WOE effectively reduced the generation of nitric oxide radicals in a concentration dependent manner. The nitric oxide scavenging activity of trolox was more effective than that of WOE.

Table 1
Scavenging activity of aqueous extracts of Welsh onion green leaves (WOE) against superoxide and hydroxyl radicals

Concentration (mg/ml)	$O_2^{-\bullet}$ (%)		·OH (%)	
	WOE	Trolox	WOE	Trolox
0.05	5 ± 1^a	14 ± 2^a	6 ± 1^a	28 ± 2^a
0.1	14 ± 1^b	25 ± 3^b	22 ± 2^b	48 ± 2^b
0.2	25 ± 2^c	37 ± 4^c	37 ± 3^c	65 ± 4^c
0.5	48 ± 4^d	64 ± 5^d	82 ± 2^d	73 ± 4^c
1.0	80 ± 1^e	87 ± 1^e	N.D.	N.D.

Superoxide radicals were generated by the NADH–phenazine methosulphate system and assayed by the reduction of NBT. Hydroxyl radicals were generated by the Fe^{3+}–ascorbate–H_2O_2 system and evaluated as TBARS levels by degradation of deoxyribose. The data are displayed as means ± SD ($n = 6$). Data in the same column marked by different superscripts are significantly different ($p < 0.05$).

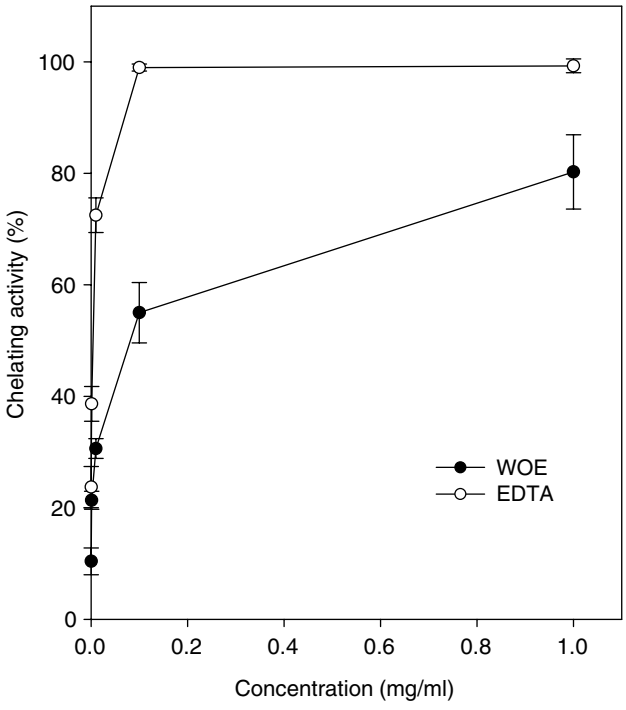

Fig. 2. Chelating effect of aqueous extracts of Welsh onion green leave (WOE) and EDTA on ferrous ions. The data are displayed as means ± SD ($n = 6$). Results were analyzed by ANOVA ($p < 0.05$).

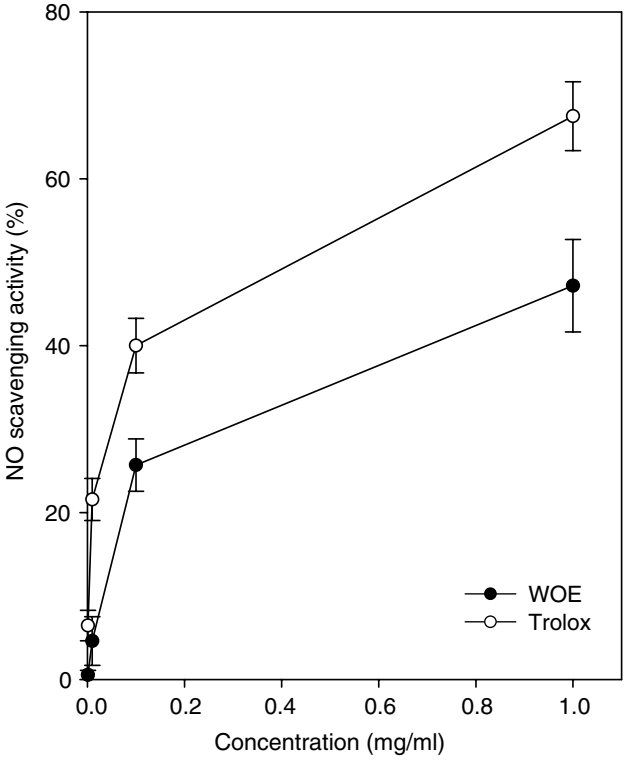

Fig. 3. Scavenging activity of aqueous extracts of Welsh onion green leave (WOE) and trolox on nitric oxide. Nitric oxide was generated by sodium nitroprusside at pH 7.4. The data are displayed as means ± SD ($n = 6$). Results were analyzed by ANOVA ($p < 0.05$).

3.4. Effect on protein oxidation induced by ·OH and HOCl

The effect of WOE on oxidative damage of albumin induced by ·OH is shown in Fig. 4. WOE showed a concentration-dependent reduction of albumin oxidation, induced by the Fe^{3+}–ascorbate–H_2O_2 system, which resulted in formation of a carbonyl group. The inhibitory activity of trolox against albumin oxidation was more obvious than that of WOE. The effects of trolox at 0.01 mg/ml reached a plateau of 70% inhibition. On the other hand, the protective effect of WOE on oxidative damage of albumin induced by HOCl is shown in Fig. 5. WOE, in the range 0.5–2 mg/ml, showed a concentration-dependent inhibition of protein oxidation induced by HOCl.

3.5. Effect on protein tyrosine residue nitration in mouse heart homogenate

Fig. 6 shows the protein tyrosine residue nitration level, measured as nitrotyrosine by immunoblot, in mouse heart homogenate incubated with horseradish peroxidase (HRP), sodium nitrite (NO_2^-) and HOCl at 37 °C for 6 h. The band signal for nitrotyrosine levels was obviously increased at 31–55 and 66–205 kDa protein (lane 2). Lanes 3–5, show that the samples treated with WOE, exhibited a concentration-dependent decrease compared to the control treated without WOE (lane 2), but their effect was inferior to the

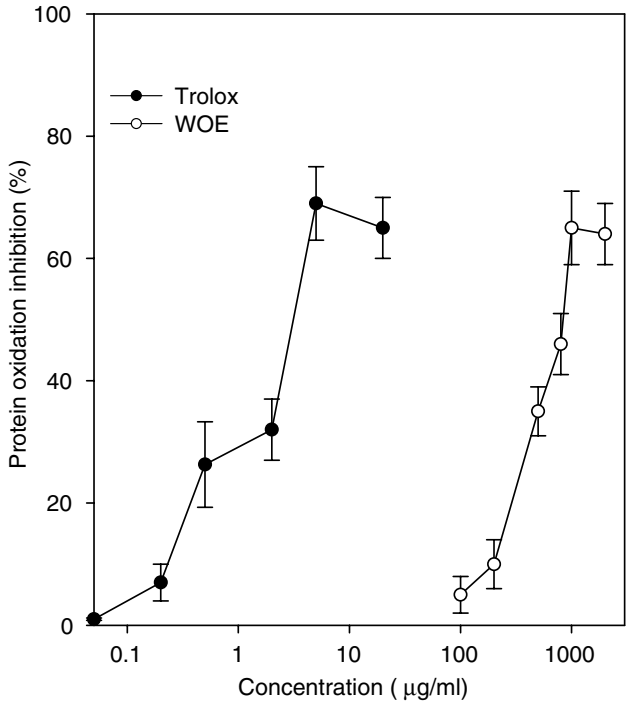

Fig. 4. Inhibitory effect of aqueous extracts of Welsh onion green leaves (WOE) and trolox on protein oxidation induced by the Fe^{2+}–ascorbate–H_2O_2 system. Extent of bovine serum albumin (BSA) carbonylation was measured as described in Section 2. Bars represent means ± SD ($n = 5$). Results were analyzed by ANOVA ($p < 0.05$).

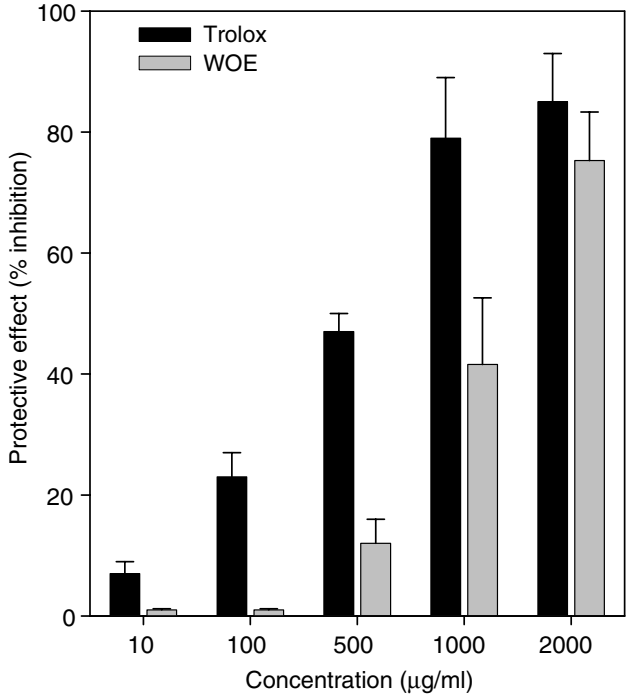

Fig. 5. Inhibitory effect of aqueous extracts of Welsh onion green leaves (WOE) and trolox on protein oxidation induced by HOCl. Extent of bovine serum albumin (BSA) carbonylation was measured as described in Section 2. Bars represent means ± SD ($n = 5$). Results were analyzed by ANOVA ($p < 0.05$).

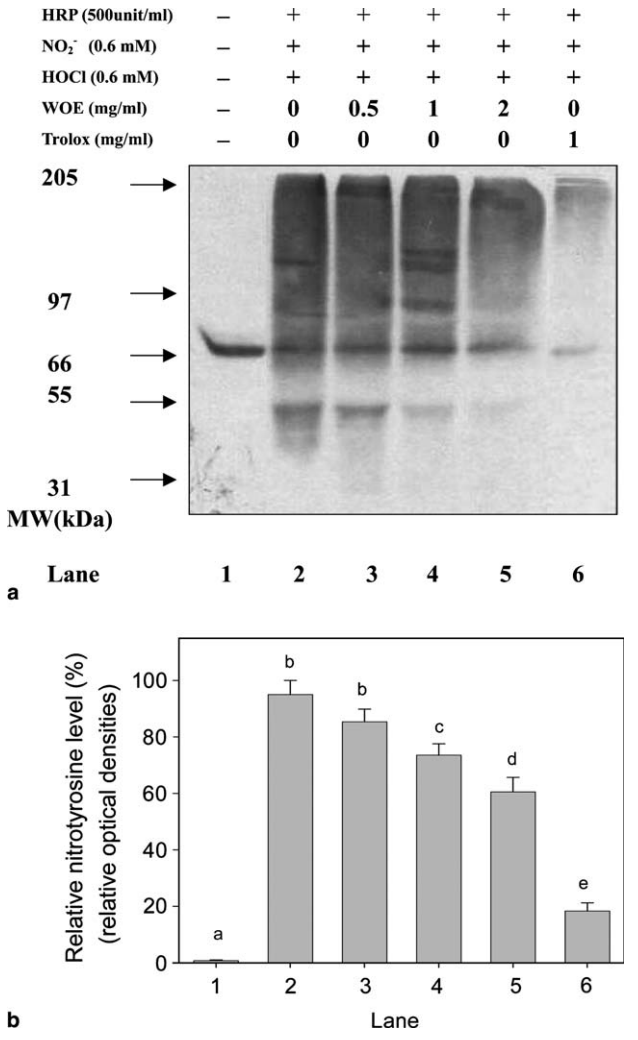

Fig. 6. Effects of aqueous extracts of Welsh onion green leaves (WOE) and trolox on protein nitration induced by the horseradish peroxidase (HRP)/NO_2^-/HOCl system. After incubation, heart homogenates were subjected to SDS–Gel electrophoresis, followed by anti-nitrotyrosine immunoblot analysis. (a) One representative blot of three independent experiments is shown. (b) The densities of nitration protein detected per lane were normalized using tubulin protein intensities. Bars represent means ± SD of three different samples. Bars marked by different letters are significantly different ($p < 0.05$).

1.0 mg/ml trolox treatment (lane 6). This result implied that Welsh onion clearly decreased protein tyrosine residue nitration.

4. Discussion

Our results reveal that WOE inhibited xanthine oxidase activity, and scavenged superoxide and hydroxyl radical production dose-dependently (Fig. 1 and Table 1). It is well known that xanthine oxidase, not only catalyzes the oxidation of xanthine to produce superoxide stress in organisms (Chiricolo, Tazzari, Abbondanza, Dinota, & Battelli, 1991) but also plays a central role

in the process of injury that occurs upon reperfusion of ischemic cells and tissues (Matsumura et al., 1998). Iio, Ono, Kai, and Fukumoto (1986) have suggested that quercetin, kaempferol, myricetin, chrysin and morin are potent inhibitors of xanthine oxidase. Quercetin and kaempferol were reported by Chen and Tsai (1999) as the two major antioxidants in WOE. Chang et al. (1994) noted that naturally-occurring phenolics, such as caffeic acid, exhibited obvious inhibitory effects on xanthine oxidase. Ou et al. (2003) noted that four organosulfur compounds (diallyl sulfide, diallyl disulfide, *S*-ethylcysteine and *N*-acetylcysteine) derived from garlic significantly inhibited xanthine oxidase. These data imply that the WOE inhibitory effects on xanthine oxidase may be a result of quercetin, kaempferol and some unknown bioactive compounds present in WOE. Therefore, suppression of the superoxide radical, as was seen in this study, by WOE, either indirectly (Fig. 1) or directly (Table 1), probably contributes to an effective defence mechanism in the living organism suffering superoxide stress. It is notable that, at 1 mg/ml, WOE and trolox showed similar direct radical-scavenging activities (Table 1). This reveals that WOE might contain unknown bioactive compounds, which effectively act as direct superoxide scavengers.

Except for superoxide, we also examined the inhibitory action of WOE against deoxyribose degradation, which gives an indication of hydroxyl radical scavenging action (Gutteridge & Halliwell, 1988). One of the commonly accepted in vivo mechanisms in the presence of transition metals,such as Fe^{2+}, is referred to as the superoxide-driven Fenton reaction (Gutteridge, 1985). Hydroxyl radicals can promote adverse and irreversible oxidations of biomolecules (e.g., protein and lipid), or can initiate chain reactions for propagating radical regeneration. Therefore, WOE shows excellent hydroxyl radical-scavenging activities (Table 1) and could be proposed to confer effective protection against this cytotoxic oxidation damage by scavenge of the initiating radical. However, it is also likely that the inhibition of the iron-dependent (Fe^{3+}/ascorbate) lipid peroxidation may result from metal chelation, a characteristic feature of many natural polyphenolic compounds. Recently, oral iron chelators have been used to treat patients with cardiovascular disease (Duffy et al., 2001) and atherosclerosis (Matthews et al., 1997).

Although WOE showed good iron chelating activity (Fig. 2), whether WOE could reduce metal toxicity in human metal intoxication, or not, is not clear. Thus, further study is needed to clarify this point. Besides these ROS, RNS (e.g., NO and peroxynitrite) have been suggested to play a causative role in the progression of many diseases. It is well known that NO reacts with superoxide, yielding the more toxic peroxynitrite ($ONOO^-$) radicals. Except directly decreasing NO, it also seems possible that some components of WOE could interfere with the SNP reduction reaction in the SNP redox cycle rather than cause direct NO scavenging effects. Under physiological conditions, peroxynitrite formation occurs only if nitric oxide is produced in high enough concentrations to overcome endogenous antioxidants. Micromolar concentrations of NO, as produced by iNOS rather than by the constitutive endothelial NOS (eNOS), are necessary for effective for peroxynitrite formation. Previous studies have suggested that garlic attenuates NO production by cytokine or LPS as stimuli (Dirsch, Kiemer, Wagner, & Vollmar, 1998). A previous study (Wang et al., 2005) has reported that WOE inhibited iNOS protein production in LPS-stimulated RAW 264.7 macrophages. These results implied that WOE might inhibit RNS stress by decreasing NO production, by direct or indirect pathways.

According to Fig. 1, the antioxidant activity of WOE correlated well with flavonoid contents. In addition, the scavenging of superoxide may, in part, contribute to the antioxidant activity. As such, these flavonoid contents show (with correlated trend) concentration-dependent antioxidant activities present in WOE. The scavenging activities of WOE against superoxide, hydroxyl radical and nitric oxide are obviously attributable to the flavonoid contents.Many reports show that flavonoids are able to scavenge superoxide anions (Tsujimoto, Hashizume, & Yamazaki, 1993) and the nitric oxide radical (van Acker, Tromp, Haenen, van der Vijgh, & Bast, 1995). These polyphenolic compounds possessed redox activity, which allow themselves to act as reducing agents, hydrogen donators and free radical-scavengers (Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995). Miean and Mohamed (2001) have reported abundant polyphenolic compounds in onion leaves. Quercetin (130 mg/kg) and kaempferol (90 mg/kg) have been identified in WOE (Chen & Tsai, 1999). In addition, quercetin and kaempferol have been suggested to have significant anti-oxidant and anti-inflammatory activities. Consequently, it is possible that those flavonoid compounds, present in the WOE, were the main contributors to the antioxidant activity. However, the antioxidant activities of flavonoids have been reported to mainly depend on the B-ring hydroxyl group number and configuration. And, the metal-chelating ability of flavonoids can form between the 5-OH and 4-oxo groups, or between the 3′-OH and 4′-OH groups (Heim, Tagliaferro, & Bobilya, 2002). Therefore, components in WOE able to inhibit hydroxyl radical and metal ions could also involve these mechanisms.

Furthermore, proteins, important components of cell and tissue, are susceptible to oxidation by ROS and RNS (e.g., $^{\cdot}OH$, HOCl and $ONOO^-$). The most common method for determination of protein oxidation is to evaluate the levels of carbonyl group, a stable product of protein oxidation, by reacting with 2,4-dinitrophenyl hydrazine to form a hydrazone chromophore.

Furthermore, nitrotyrosine has been proposed to be another specific marker of protein tyrosine oxidized and tissue injured by RNS in vivo. Eiserich et al. (1998) have demonstrated that NO_2^-, a major end-product of NO metabolism, readily promotes tyrosine nitration, through formation of nitryl chloride (NO_2Cl) and nitrogen dioxide ($^{\cdot}NO_2$), by reaction with the inflammatory mediators, hypochlorous acid (HOCl) or myeloperoxidase. Sampson et al. (1998) have also suggested that peroxidase-catalyzed nitration of tyrosine could occur in the presence of competing substrates in vivo. These reports have implied that the $HRP/NO_2^-/HOCl$ system is a possible source of reactive nitrogen species in complex biological mixtures. In Figs. 4 and 5, WOE clearly decreased protein carbonyl derivatives production (from protein oxidation induced by $^{\cdot}OH$ and HOCl). Also, in Fig. 6, WOE showed a protective role in mouse heart homogenate protein tyrosine residue nitration induced by reactive nitrogen species. Selloum, Djelili, Sebihi, and Arnhold (2004) have suggested that flavonols, rutin, quercetin and kaempferol, could obviously scavenge HOCl. In addition, Binsack et al. (2001) noted that 6-chloroquercetin and 6,8-dichloroquercetin, derived from the reaction between HOCl and quercetin, were more potent antioxidants toward oxidative modification of low-density lipoproteins and ABTS radical formation than the unmodified form. Furthermore, Haenen, Paquay, Korthouwer, and Bast (1997) have reported that quercetin was the most potent flavonoid in scavenging $ONOO^-$, and the catechol group and the hydroxyl group at position 3 give the highest contribution to the $ONOO^-$-scavenging effect. Since quercetin and kaempferol in WOE have been identified (Chen & Tsai, 1999), we suggest that the flavonoids in WOE, such as quercetin and kaempferol, might contribute to protect protein tyrosine residue nitration as a result of scavenging of $ONOO^-$. Thus, this result implies that WOE, with quercetin, kaempferol and other active flavonoids, are significantly helpful to consumers to protect against pro-inflammatory stress.

In conclusion, the fact that WOE protected protein from oxidation may be attributed to their scavenging of reactive oxygen and nitrogen species. These findings imply Welsh onion might contain effective antioxidants against the oxidative damage and serious threat of various conditions. Further research is still needed to investigate the nutritional and physiological effects of Welsh onion in vivo in more detail.

Acknowledgements

This work was supported by a grant from the National Science Council of the Republic of China (NSC 92-2320-B-041-004) and Chia Nan University (CNAL-92-04).

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