行政院國家科學委員會專題研究計畫　成果報告

青蔥萃取物影響巨噬泡沫細胞基質分解活性之研究

計畫類別：個別型計畫
計畫編號：NSC93-2320-B-041-005-
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計畫主持人：王柏森

計畫參與人員：林姿吟 凌秀華 陳慧如

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計畫參與人員：林姿吟　　嘉南藥理科技大學　食品科技系
凌秀華　　嘉南藥理科技大學　生活應用與保健系
陳慧如　　嘉南藥理科技大學　生活應用與保健系

執行單位：嘉南藥理科技大學

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中　華　民　國　　94 年　10 月　28 日

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 of 0.05 – 1.0 mg/ml showed a potent concentration-dependent manner in reducing xanthine oxidase activity, and this inhibitory actions of WOE correlated well with total flavonoid content (r= 0.99). Furthermore, WOE also showed scavenging superoxide radical, hydroxyl radical, nitric oxide and chelating metal ions 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 WOE . Protein tyrosine residue nitration in mouse heart homogenates was obviously decreased by 2 mg/ml WOE. These findings revealed that WOE might show in vitro antioxidant activity by their directly scavenging reactive oxygen and nitrogen species, inhibiting xanthine oxidase and chelating metal ions. Thus, these positive regulations might contribute to the protective effect of Welsh onion on oxidative damage of protein in vitro.

Keywords: Welsh onion; antioxidant; reactive oxygen species; nitric oxide; protein oxidation

二、緣由與目的

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 some antioxidants present including vitamins (Palli et al., 2003), carotene (Nishino, 1998) and flavonoids (Rossi et al., 2003). And, these chemoprotective reactions were made through scavenging free radicals (Aldini, Carini, Piccoli, Rossoni & Facino, 2003), chelating trace metal elements (e.g. iron and copper) (Borsari et al., 2001) or inhibition some enzymes involved in formation of free radicals (e.g. xanthine oxidase, lipooxygenase, NADPH oxidase and cyclooxygenase) (Lampe, 1999). In the opinion of these reports, the inhibition or scavenging activities on free radicals have been proposed as a protective power in evaluating on our diet. Therefore, increasing ingestion of such diets may offer in the belief to help in maintaining good health. However, under physiological conditions, there is a continuous production of free radicals such as superoxide radicals, hydroxyl radicals and nitric oxide. These reactive species are essential for life at lower quantities, because they are involved in cell signaling and physiological processes. In addition to these necessary functions, nonessential production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) react with cell molecules, induce protein oxidation and responsible for harmful damage in cells and tissues where there were generated. For example, neutrophils and macrophages could generate mass superoxide or NO in response to different extracellular stimulants (e.g. lipopolysaccharide and cytokines). 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 for different dietary antioxidants to help prevent development of oxidative damage (Borek, 2001; Rao, 2002). Welsh onion (*Allium fistulosum* L., Alliaceae), an important flavoring vegetable in Asian dishes, has been reported to inhibit LDL oxidation (Wang et al, 2005), modulate aortic vascular tone (Chen, Tsai & Chen, 1999) and lower blood pressure (Chen, Chen, Tsai & Jen, 2000).

Other members of *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). There is a positive correlation between related aging diseases and protein oxidation in tissue. Protein oxidation resulted in protein denaturation and loss of their function, which was regarded as a marker of tissue damage and aging. Consequently, inflammatory processes, atherosclerosis and cancer are concomitant with the development of protein oxidation (Cuzzocrea, Riley, Caputi & Salvemini, 2001). Although, in our previous study, Welsh onion has been known 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 the reactive oxygen and nitrogen species were investigated. The anti-protein oxidation by Welsh onion was also evaluated.

三、結果與討論

3.1.Inhibitory effect on xanthine oxidase and total flavonoid content

Figure 1 showed the inhibitory effect of WOE on xanthine oxidase activity and their total flavonoid content. In the range of 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). Furthermore, the inhibitory activity on xanthine oxidase by WOE depended on its concentration and correlated well (r = 0.99) with their flavonoid content.

3.2. Scavenging superoxide radicals and hydroxyl radicals production

The superoxide scavenging activity was assayed by NADH-PMS-NBT system (Figure 2). The result showed scavenging superoxide activity increased with increasing concentration of WOE and Trolox. In the range of 0.05-1.0 mg/ml, WOE and Trolox showed the percentage of scavenging activity on superoxide was 5.0 – 80.0 % and 14.0 – 87.0 % in NADH-PMS-NBT model system, respectively. These results revealed that WOE possesses scavenging effect on superoxide. Figure 3 shows the hydroxyl radical scavenging activity of WOE. The hydroxyl radicals generated from Fe^{3+}-ascobate-H_2O_2 system caused deoxyribose degradation to TBARS. In the range of 0.1 – 0.5 mg/ml of WOE, TBARS production was decreased with increasing concentration of WOE. Comparing to the Trolox, the scavenging activity of WOE on hydroxyl radical in the range of 0.05 – 0.2 mg/ml is inferior to Trolox.

3.3. Chelating metal ion and scavenging nitric oxide

The chelating activity of WOE was evaluated by means of ferrozine assay, and the results were showed in Figure 4. The chelating effects of WOE on ferrous ions increased with increased concentrations. One mg/ml of WOE exhibited 80% chelating effects on ferrous ions. Certainly, the chelating effects 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 on nitric oxide by WOE on sodium nitroprusside (SNP) system is shown in Figure 5. 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 with a concentration dependent manner. The nitric oxide scavenging activity of Trolox was more effective than that of WOE.

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

The effects of WOE on oxidative damage of albumin induced by ·OH was shown in Figure 6. WOE showed a concentration-dependent fashion in reducing albumin oxidation induced by Fe^{3+}-ascobate-H_2O_2 system, which resulted in

formation of carbonyl group. The inhibitory activity of Trolox on albumin oxidation was more obvious than WOE. The effects of Trolox at 0.01 mg/ml reached a plateau of 70 % inhibition. On the other side, the protective effect of WOE on oxidative damage of albumin induced by HOCl was shown in Figure 7. WOE in the range of 0.5 – 2 mg/ml showed a concentration dependent manner in inhibitory effect on protein oxidation induced by HOCl.

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

Figure 8 showed 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 degrees Celsius for 6 hr. The band signal present nitrotyrosine levels was obviously increased at 31 – 55 and 66 - 205 kDa protein (lane 2), respectively. Lanes 3 – 5, the samples treated with WOE, exhibited a concentration dependent decrease comparing to the control treated without WOE (lane 2), but their effect was inferior to 1.0 mg/ml Trolox treatment (lane 6). This result implied that Welsh onion clearly decreased protein tyrosine residue nitration.

ROS and RNS involved in different pathological processes, such as cardiovascular diseases, atherosclerosis and inflammation have attracted much concern. Therefore, how to decrease these oxidative stresses and restore intracellular antioxidant capacity has been thought to be an important goal in promoting health. However, the use of *Allium's* vegetables present a large source of natural antioxidants has 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 suggested that these beneficial effects are mainly attributed to their organosulfur compounds. These 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).

Our results revealed that WOE dose-dependently inhibited xanthine oxidase activity, scavenged superoxide and hydroxyl radical production (Figures 1, 2 and 3). It is well known that xanthine oxidase not only catalyzed the oxidation of xanthine to produce superoxide stress in the organisms (Chiricolo, Tazzari, Abbondanza, Dinota & Battelli, 1991) but also played a central role in the process of injury that occurs upon reperfusion of ischemic cells and tissues (Matsumura et al., 1998). Therefore, suppression of the superoxide radical as was seen in this study by WOE, either indirectly (Figures 1) or directly (Figure 2), is probably one effective defense mechanism in living organism against superoxide stress. One of the most noticeable is, at 1 mg/ml, WOE and Trolox showed a similar direct radical scavenging activity (Figure 2). This data reveal that WOE might contain unknown compounds, which effectively act as a direct superoxide scavenger.

Except superoxide, we also examined the inhibitory action of WOE on deoxyribose degradation, which gives an indication of hydroxyl radical scavenging action (Gutteridge & Halliwell, 1988). One of the common accepted concepts of hydroxyl radical, formed in vivo by the interaction of hydrogen peroxide with superoxide if the presence of transition metals such as Fe^{2+} can be demonstrated, a reaction sequence referred to as the superoxide-driven Fenton reaction (Gutteridge, 1985). Hydroxyl radical can promote adverse and irreversible oxidations of biomolecules (e.g. protein and lipid), or it can initiate chain reactions of propagating radical regeneration. Therefore, WOE showed here an excellent hydroxyl radical scavenging activities (Figure 3) and could be proposed to be effective protection against this cytotoxic oxidation damage through to scavenge 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 (Figure 4), whether WOE could reduce metal toxicity in human metal intoxication or not is not clear. Thus, further study 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 react with superoxide, yielding more toxic peroxynitrite ($ONOO^-$) radicals. Except directly decrease NO, it also seems to be possible that some components of WOE could interfere SNP reduction reaction in SNP redox cycle rather than direct scavenging NO effects. Under physiological conditions, peroxynitrite formation occurs only if nitric oxide is produced in high enough concentrations to outcome 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 attenuated NO production by cytokine or LPS as stimuli (Dirsch, Kiemer, Wagner & Vollmar, 1998). In previous study (Wang et al., 2005) has reported that WOE inhibited iNOS protein production in LPS-stimulated RAW 264.7 macrophages. These data implied WOE might inhibit RNS stress by decreasing NO production by directly or indirectly pathways.

According to the data presented in Figure 1, the antioxidant activity of WOE correlated well with flavonoid contents. The flavonoid content in WOE increased with an increased concentration of WOE (Figure 1). In addition, the scavenging of superoxide may in part contribute to the antioxidant activity. As such, these flavonoid contents present with correlated trend in a concentration dependent manner with their antioxidant activities present in WOE. Comparing to the results of scavenging action of WOE on superoxide and the correlation between the flavonoid contents and the WOE concentration ($r = 0.99$), the scavenging activity of WOE on superoxide, hydroxyl radical and nitric oxide may obviously attribute to the flavonoids contents. Admittedly, many literatures reported that flavonoids belonging to polyphenolic compounds 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 made themself to act as reducing agent, hydrogen donators and free radicals scavenger (Rice-Evans, Miller, Bolwell, Bramley & Pridham, 1995). Miean and Mohamed (2001) have reported that the abundant polyphenolic compounds were found in onion leaves. Chen and Tsai (1999) have identified quercetin (130 mg/kg) and kaempferol (90 mg/kg) in WOE. Quercetin and kaempferol (Figure 9) have been suggested to have significantly anti-oxidant and anti-inflammatory activity. Consequently, it is possible that those flavonoid compounds present in the WOE were the mainly contributing 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, the possible structure properties of some components in WOE to inhibit hydroxyl radical and metal ions could be also based on these mechanisms.

Furthermore, proteins, the 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 protein oxidation is to evaluate the levels of carbonyl group, a stable product after 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 (・NO_2) by reaction with the inflammatory mediators hypochlorous acid (HOCl) or myeloperoxidase. Sampson et al. (1998) also have suggested that peroxidase-catalyzed nitration of tyrosine could occur in the presence of competing substrates in vivo. These reports implied that HRP/ NO_2^-/HOCl system is a possible source of reactive nitrogen species in complex biological mixture. In Figure 6 and Figure 7, WOE clearly decreased protein carbonyl derivatives production from protein oxidation induced by ・OH and HOCl. Also, in Figure 8, 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 have been identified in WOE (Chen & Tsai, 1999). We suggested that the flavonoids in WOE such as quercetin and kaempferol might contribute to protect protein tyrosine residue nitration as a result of scavenging $ONOO^-$. Thus, this result implied that WOE with quercetin, kaempferol and other active flavonoids is significantly meaningful to consumer because of their against proinflammatory stress.

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

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