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Photodynamic Effects of Methionine-Riboflavin Mixture on Antioxidant Proteins

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ABSTRACT

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Photodynamic action of methionine-riboflavin mixture (MR) with a high level of reactive oxygen species (ROS) production is a powerful microbicidal system. Four important antioxidant proteins, including catalase, peroxidase, superoxide dismutase (SOD) and bovine serum albumin (BSA), were used to study the effects of MR photodynamic action on protein structure and enzymatic activity and to reveal the functional roles of ROS in MR toxicity. Protein structure damages by MR included degradation and cross-linking. All tested proteins were affected by MR and the level of degradation augmented as the length of the illumination treatment increased. Larger proteins were formed for catalase and SOD due to cross-linking following the MR treatment. Enzymatic activities of catalase, peroxidase and SOD decreased significantly in MR treatment at very early stage (15 min after illumination). Among the four proteins, SOD was the least sensitive protein to MR on both protein degradation and enzymatic activity reduction. Using ROS scavengers and ROS production promoters, ROS were demonstrated to be required for protein damages by MR. The damages of cellular important antioxidant proteins at a very early stage may result in the failure of cellular defense systems to oxidative stress and thus lead to cell death by MR.

Key words: catalase, peroxidase, superoxide dismutase, bovine serum albumin (BSA), antioxidants, oxidative stress, protein degradation, cross-linking

INTRODUCTION

Photodynamic action of methionine riboflavin mixture (MR) is a powerful microbicidal system. It consists of two naturally produced compounds: methionine and riboflavin. Under illumination, riboflavin could be excited by light to a triplet state and thereafter activate oxygen and methionine in the mixture by energy transfer. This activation process thus produces an abundant amount of toxic materials and incurs a strong biocidal activity. In previous studies, we have shown that MR photodynamic action is very toxic to a wide spectrum of organisms, including various phytopathogenic fungi, bacteria and viruses [26, 28, 30, 33]. Under field conditions, sunlight can trigger the photodynamic action of MR and control plant diseases caused by fungi [26, 34]. A formulation based on MR has been commercialized by Taiwan Cyanamide Co. and used to control powdery mildew in crop plants in Taiwan for a period of time. Additional to powdery mildew, the MR based formulation was recently showed to control flyspeck and sooty blotch of apple and pear in Wisconsin, USA [1].

The reaction mechanism of MR photodynamic action

has been studied well in our laboratory. By using electron spin resonance spectroscopy, we have detected large amount of reactive oxygen species (ROS) and shown that the production of ROS is closely related to microbial toxicity of MR photodynamic action [31, 32]. Those ROS include superoxide anion, hydrogen peroxide, singlet oxygen and hydroxyl free radical [27, 29, 31, 32].

Many studies regarding ROS in relation to biology and medicine have been published. ROS can be endogenously formed in a biological cell. For example, superoxide anion and hydrogen peroxide are both produced during the electron transfer in oxidative respiration, while singlet oxygen is associated with photosynthesis. ROS can cause aging and several human diseases [4, 6, 11, 12, 17, 25]. On the other hand, phagocyte superoxide generation plays a key role in the killing of some bacterial strains in the human immune system [8, 22]. However, the biological cell has various detoxification mechanisms to defend themselves from the damage of the toxic ROS [18, 25]. Biological enzymes, such as superoxide dismutase (SOD), catalase, and peroxidase, provide the first line of antioxidant defense in cells. SOD converts superoxide anion to weaker toxic hydrogen peroxide. Both catalase and peroxidase are able to remove hydrogen peroxide subsequently. Other detoxifying molecules such as α-tocopherol and β-carotene are important biological antioxidants. α-tocopherol (vitamin E) is the major scavenger inside membrane systems of cells [9, 19], while β-carotenoids deprive the singlet oxygen during photosynthesis and are also capable of peroxy radical quenching [5, 23]. Additionally, both transferrin and serum albumin proteins bind with free form of both iron and copper ion to prevent the hydroxyl free radical production via metal-catalyzed Haber-Weiss reaction [11, 12].

Cellular antioxidant proteins such as SOD, catalase and peroxidase would be very important to microbes to defend themselves from the damages caused by MR photodynamic action. However, most tested microbes can not survive well under MR photodynamic action. Therefore, we hypothesized that MR photodynamic action can damage cellular antioxidant proteins at an early stage and then results in rapid cell death. To test this hypothesis, the effects of MR photodynamic action on antioxidant protein structures and enzymatic activities were investigated. In this study, we report on the damages to antioxidant proteins by MR photodynamic action and discuss on the roles of ROS in the damages.

MATERIALS AND METHODS

Chemicals and Reagents

Riboflavin, methionine, α-tocopherol, β-carotene, butylated hydroxyanisole (BHA), bovine serum albumin (BSA), nitro blue tetrazolium (NBT), xanthine, xanthine oxidase and peroxidase (type I) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Superoxide dismutase (SOD, from beef liver; EC 1.15.1.1) and catalase (from beef liver; EC1.11.1.6) were purchased from Boehringer Mannheim Chemical Co. (Mannheim, Germany). Chemicals used for polyacrylamide gel electrophoresis were from Bio-RadLaboratories (Richmond, California, USA). Chemicals used in this study and not mentioned above were purchased from Merck Chemical Co. (West Germany). All the chemicals used were reagent grade or equivalent in purity. Freshly made glass double distilled water was used for the preparation or dilution of all the reaction mixtures.

MR photodynamic action

In all tests, MR was made freshly with glass double distilled water, containing 26.6 μM riboflavin and 1 mM methionine. Unless specified, pH of MR was adjusted to 4.0 with 100 mM citrate phosphate buffer. MR photodynamic action under light illumination was performed as described previously [14]. Experiments were conducted with at least three replicates of each treatment within an experiment and all experiments were repeated at least once with similar results.

Protein degradation assays

Bovine serum albumin, peroxidase, catalase or superoxide dismutase was added into MR to 1 mg/ml of final concentration and performed illumination immediately. During the time course experiment, aliquots were removed from the MR-protein reaction mixture and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [13]. MR treated protein solutions were mixed immediately with SDS-PAGE sample degrading buffer [13] and then heated for 2 min at 100℃. Protein subunits were separated by 15% of SDS-PAGE and the gel was developed by silver staining as described by Chuang [13].

Enzyme activity assays

To determine the effect of MR photodynamic action on the activities of ROS detoxification enzymes, catalase, peroxidase or SOD, was added into 1ml of MR and the MR-enzyme mixture was immediately placed under the light treatment. The final concentration of each enzyme in MR was 0.2 mg/ml for catalase, 0.1 mg/ml for peroxidase and 0.1 mg/ml for SOD. During the time course experiment with illumination, enzyme activity was assayed immediately as described below. All reactions were conducted using a Shimadzu UV-160A spectrophotometer

with the cell holder temperature controlled at 25℃.

Both catalase and peroxidase activity assays were performed as described in the Worthington Enzyme's Manual [7]. To assay catalase activity, the substrate solution (59 mM H_2O_2) was prepared in 50 mM potassium phosphate buffer, pH7.0. One ml substrate solution and 1.9 ml double distilled water were added into a 1-cm cuvette, and then incubated in the spectrophotometer for 4 min to achieve temperature equilibration at 25℃. Then 0.1 ml of 10-fold diluted MR treated-catalase mixture was added into the cuvette and mixed well by pipetting the solution up and down. The decrease of absorbance at 240 nm was recorded for 2-3 min. The linear portion (ΔA_{240}/min) of the reaction curve was used to calculate specific activity. The substrate solution (1.7 mM) for the peroxidase activity assay was prepared by diluting H_2O_2 with 0.2 M potassium phosphate buffer, pH7.0. 4-aminoantipyrune was used as an electron donor and its working solution (2.5 mM) was made by dissolving this chemical into 0.17 M phenol. Peroxidase activity detection was performed using a spectrophotometer. A 1-cm cuvette containing 1.5 ml substrate solution and 1.4 ml 4-aminoantipyrune solution was incubated in the spectrophotometer at 25℃ for 4 min. Then 0.1 ml of 10-fold diluted MR -peroxidase solution was added and ΔA_{510}/min was recorded. The specific activities of peroxidase and catalase were calculated as described in the Worthington Enzyme Manual [7].

Superoxide dismutase activity was detected as described by Beauchamp and Fridovich [3]. Reaction mixture containing 1 ml of 0.13 mM xanthine, 1.6 ml of 0.1 M potassium phosphate buffer (pH 7.8) with 0.187 mM EDTA, 0.2 ml of 1.5 mM NBT, and 0.1 ml MR-SOD solution were placed in a 1-cm cuvette, and then incubated in the spectrophotometer at 25℃ for 4 min. To start the reaction, 0.1 ml of 0.15 units/ml xanthine oxidase was added into the cuvette and A_{560} was recorded for 4 mins. The specific activity was calculated by the formula as: percent inhibition (%) = (Aw-As)/Aw X 100% in which "Aw" is the ΔA_{560}/min of control (water) and "As" is the ΔA_{560}/min of the tested SOD solution.

pH and antioxidant treatments

BSA (1 mg/ml) was treated with the MR containing α-tocopherol, β-carotene or butylated hydroxyanisole (BHA) at 100 mg/ml each. The MR containing 5 % ethanol was used as control treatment since the stock solutions of β-carotene and BHA were prepared with ethanol. For the pH effects, MR-mixture was adjusted to pH 4.0, 5.0, 6.0, 7.0 or 8.0 with 0.01 M citrate phosphate and/or phosphate buffers. BSA (1 mg/ml) and $FeCl_2$-EDTA (1 mM-100 mg/ml) were added into pH adjusted-MR solutions and immediately conducted with illumination. $FeCl_2$-EDTA complexes are excellent promoters for hydroxyl radical formation via Haber-Weiss reaction [2, 11]. The concentration of iron-EDTA used in this test was suggested by previous studies in our laboratory.

RESULTS

The influence of MR photodynamic action on the structures of antioxidant proteins

Biologically antioxidant proteins including BSA, peroxidase, catalase and SOD were treated with MR under continuous illumination and then analyzed by 15 % SDS-PAGE with silver staining. The results showed that MR treated proteins are all degraded (Fig. 1-4).

After analysis by SDS-PAGE and silver stain, the major protein bands of BSA (indicated by arrows in Fig. 1) were degraded following the MR treatment. The level of degradation augmented as the time of illumination treatment increased. The three major bands of BSA were greatly diminished after incubation in MR for 8 hr. On the other hand, the amount of smaller polypeptide fragments shown as smear increased significantly 1 hr after treatment compared to those processed in distilled water, and their amount grew as time of treatment increased. The density of smear patterns increased as the density of the major subunit proteins decreased. It indicated that molecules in the smear were derived from the degradation of major subunit protein (Fig. 1).

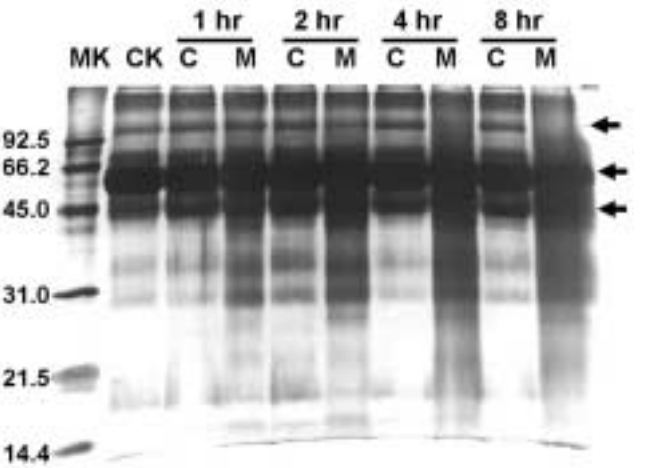

Fig. 1. Damage of BSA protein by MR. Proteins were analyzed by SDS-PAGE and silver stain after1, 2, 4, and 8 hr illumination. Three major bands of BSA are indicated by arrows. MK, protein molecular markers; CK, control protein without any treatment; C, control treatment under illumination; M, MR treated under illumination. Size is in kDa.

Three tested enzymes proteins were also degraded significantly during the process of MR-treatment. As shown in Figure 2, the 62.5 kDa main subunit protein of catalase was almost degraded completely by MR 8 hr after continuous illumination. In MR treatments, the degradation products in the smear appeared notably 2 hr and increased 4 hr after illumination. At the same time, a polypeptide larger than the 62.5 kDa main subunit protein was formed in MR treatment and the amount increased 4 hr after treatment (indicated by hollow triangles in Fig. 2). Furthermore, another protein larger than 92.5 kDa was detected 4 and 8 hr after illumination (indicated by hollow arrows in Fig. 2). The same phenomenum was observed with SOD and peroxidase (Figs. 3 and 4). As for SOD, two small polypeptides were formed 2 hr after illumination and incresed as illumination time went on (indicated with arrow head in Fig.3). A band slightly larger than the 16.0 kDa SOD subunit protein was also formed indistinctly (indicated by arrows in Fig. 3). For peroxidase, the phenomenon of the protein being degraded became obvious as the MR-treatment extended. Its four major subunit proteins could not be clearly identified 8 h after the treatment (indicated by arrows in Fig.4).

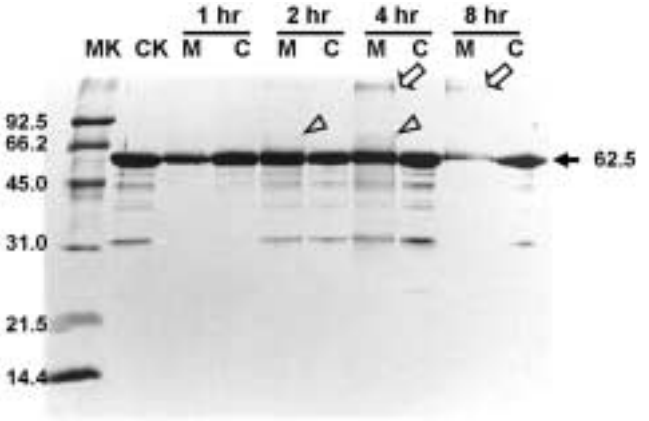

Fig. 2. Damage of catalase protein by MR. Proteins were analyzed by SDS-PAGE and silver stain after 1, 2, 4, and 8 hr illumination. Two large proteins (shown with triangles and hollow arrows) were formed after treated with MR. The 62.5 kDa subunit of catalase is indicated by arrow. MK, protein molecular markers; CK, control protein without any treatment; C, control treatment under illumination; M, MR treated under illumination. Size is in kDa.

The influence of MR photodynamic action on antioxidant enzyme activity

To further understand the effects of MR photodynamic action on antioxidant enzymes, three important ROS scavengers including catalase, peroxidase and SOD were treated with MR under continuous illumination. As shown in Fig. 5, the activities of three enzymes decreased as time of treatment increased (Fig 5). Among the three, catalase is the most sensitive to the length of illumination treatment. Fifteen minutes after the beginning of the treatment, the activity of catalase has dropped 62%, and was undetectable 2 hr after (indicated with arrows in Fig. 5A). A similar situation was observed with the peroxidase as after 15 min of illumination, the activity of the peroxidase dropped by about 30 %, and was

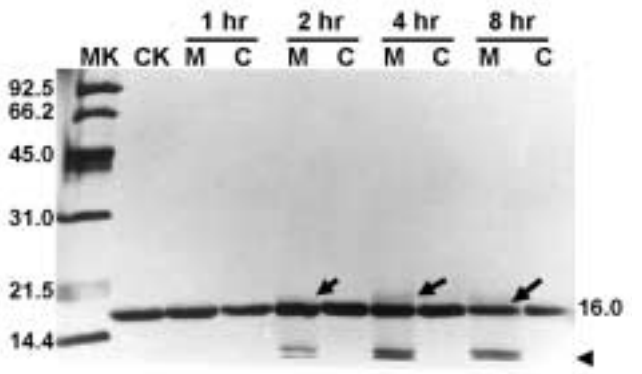

Fig. 3. Damages of SOD protein by MR. Proteins were analyzed by SDS-PAGE and silver stain after 1, 2, 4, and 8 hr illumination. A larger protein (indicated with arrows) and two small breakdown products (indicated with arrow head) were formed at 2, 4 and 8 hr after treated with MR. MK, protein molecular markers; CK, control protein without any treatment; C, control treatment under illumination; M, MR treated under illumination. Size is in kDa.

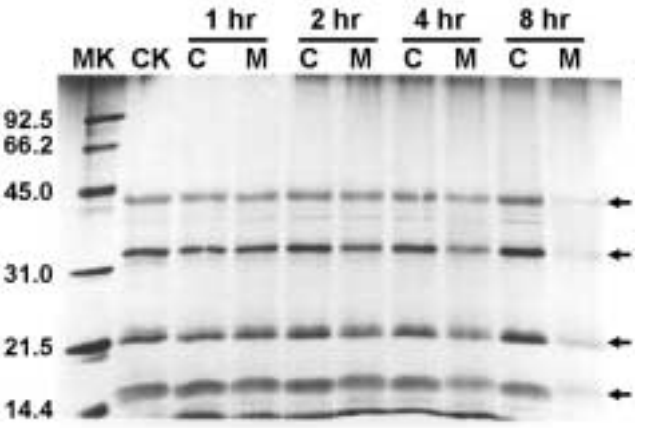

Fig. 4. Damage of peroxidase protein by MR. Proteins were analyzed by SDS-PAGE and silver stain after 1, 2, 4, and 8 hr illumination. Peroxidase four subunits are indicated with arrows. MK, protein molecular markers; CK, control protein without any treatment; C, control treatment under illumination; M, MR treated under illumination. Size is in kDa.

near zero after 2 h incubation in MR (indicated with arrows in Fig. 5B). The decrease of SOD activity under the MR-treatment was similar to what observed for peroxidase and catalase. However, SOD was less sensitive to MR photodynamic action than catalase and peroxidase (Fig. 5C). It was also found that SOD showed an extreme instability under the distilled water treatment at pH 4.0.

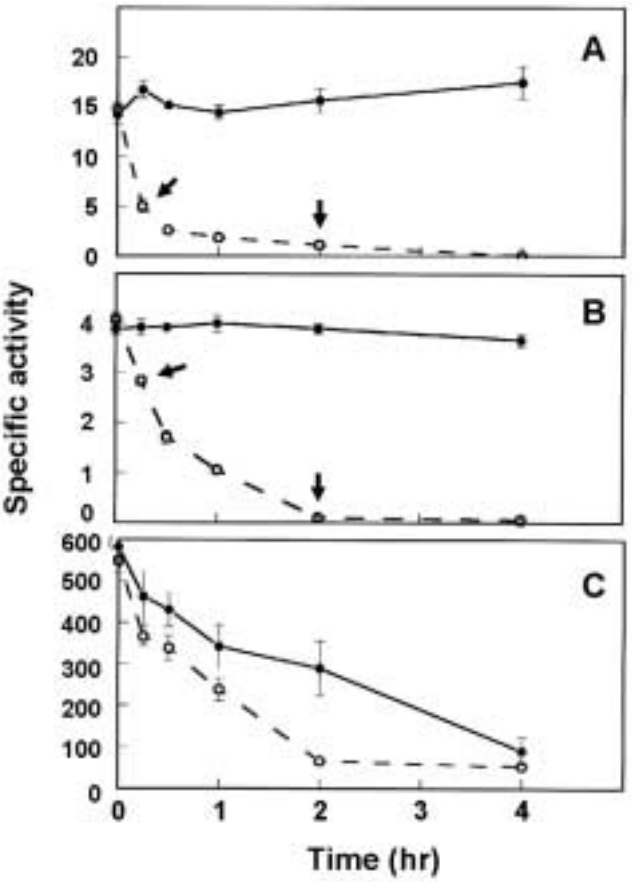

Fig. 5. The effects of MR on antioxidant enzyme activities under continuous illumination. A, catalase (units x 10^3/mg protein). B, peroxidase (units/mg protein). C, superoxide dismutase (units/mg protein). Enzyme activities were measured at 0.25, 0.5, 1, 2, and 4 hr after illumination in MR (dashed lines) and in control (solid lines). Data represent the mean and standard deviation of three separate experiments for each treatment.

The involvement of Haber-Weiss reaction in protein damages by MR photodynamic action

To investigate whether Haber-Weiss reaction was involved in protein damages under MR photodynamic action, experiments studying the effects of both iron and pH on damage to the BSA protein were conducted. In this test, BSA was treated in MR with various pHs and with ferrous ion, and then assayed by 15 % SDS-PAGE and silver stain. Four hours after illumination, the silver stain density of smaller polypeptides shown as smear had increased in all pH conditions in MR compared to those of control treatment. As time went on, the degrading phenomenon became more apparent especially at pH 5.0 as shown in Figure 6. Damage to BSA in control treatments, which were in distilled water with different pH, was not notable up to 8 h of illumination (data not shown).

The Influence of antioxidant compounds on protein damaged by MR photodynamic action

Three antioxidants including α-tocopherol and β-carotene, and BHA which is commonly used in food preservation, were used to verify the role of ROS on protein damages caused by MR photodynamic action. The amount of BSA protein decreased significantly in MR 4 hr after illumination as presented in Fig. 7. At the same time, the additions of α-tocopherol, β-carotene or BHA reduced significantly the degradation caused by MR. Among the three antioxidants, BHA was the most effective antioxidant to protect BSA from the degradation caused by MR photodynamic action (Fig. 7).

DISCUSSION

The oxidative damages on cellular lipids, proteins and DNA are central factors causing cell death by reactive oxygen species [16, 20, 21, 24, 35]. As shown in previous studies, MR photodynamic action kills many plant pathogens and produces high levels of ROS [26, 27, 28, 29, 31, 32, 33]. We have found that lipid peroxidation [14] and DNA degradation (unpublished data) are two important factors resulting in plant pathogen death by MR photodynamic action. In this study, we demonstrated that lost of enzyme activity and protein degradation of cellular important antioxidant proteins in MR may result in the failure of cellular defense systems against oxidative stress and thus leading to cell death.

The major toxic compounds produced in MR photodynamic action are ROS which are derived from oxygen during photodynamic action, mainly including hydroxyl radical, superoxide anion, singlet oxygen and H_2O_2. The production of ROS in MR and their production pathways have been well studied and discussed previously [27, 29, 31, 32]. The yields of ROS increased in MR as the illumination time increased. Among those ROS, hydroxyl free radicals are the most reactive oxygen radical known to chemistry. They have a tremendous potential to cause biological damage, since they react with all biological molecules as soon as they come in contact with them. Their production has been known to be principally by the

transient metal ion dependent Haber-Weiss reaction as shown below[2]:

$$O_2^{-\cdot} + H^+ \longrightarrow HO_2 \quad (1)$$

$$HO_2 + O_2^{-\cdot} + H^+ \longrightarrow H_2O_2 + O_2 \quad (2)$$

$$X\text{-}Fe^{+++} + O_2^{-\cdot} \longrightarrow X\text{-}F^{++} + O_2 \quad (3)$$

$$X\text{-}F^{++} + H_2O_2 \longrightarrow X\text{-}Fe^{+++} + {}^{\cdot}OH + OH^- \quad (4)$$

Superoxide anion, H_2O_2 and transient metal ion (e.g. ferrous ion) are required for hydroxyl radical production via Haber-Weiss reaction. Superoxide anion was generated when oxygen gains an electron from excited riboflavin by light in MR. In biological cell, superoxide anion can be detoxified by SOD and produce H_2O_2. However, two superoxide anions can autodismutate into one H_2O_2 when SOD is absent as shown in Haber-Weiss reaction.

In this study, all treated proteins were degraded to smaller molecular peptides when treated with MR after SDS-PAGE analysis. Among treated antioxidant proteins, SOD was the least sensitive protein to MR, not only shown on the degradation of protein structure and also shown on the loss of enzyme activity. Superoxide anions carry an unpair electron thus they are very unstable and more aggressive in attacking other molecules than H_2O_2. When SOD was treated with MR, SOD may converts superoxide anion produced in MR into less toxic H_2O_2. Therefore, SOD could be attacked by H_2O_2 and other mild radicals derived directly from riboflavin and methionine. This may be the reason that the damage to SOD was not as obvious as for the three other proteins. BSA, catalase and peroxidase are very sensitive to MR since they may be directly attacked by superoxide anions. Furthermore, all tested proteins might be attacked by hydroxyl radical produced in MR by Haber-Weiss reaction as described above due to their transient metal ion carrying characters. According to the study by Halliwell and Gutteridge[11, 12], hydroxyl radical formation is "site specific" to the points at which metal ions are located.

The role of hydroxyl radicals on protein damages was further demonstrated by ferrous ion and pH effects. The most effective pH for hydroxyl free radical production via Haber-Weiss reaction is pH 5.0 in the presence of ferrous ion. In this study, the protein degrading effect reached the highest point when the pH was 5.0 (Fig. 6). The production of hydroxyl radical in MR has been detected and showed that pH 5.0 was the best condition for hydroxyl radical production[29]. Thus, it clearly indicates that hydroxyl radical is an important factor causing protein damages in MR.

Singlet oxygen is also generated in MR, significantly at low pH[31]. The importance of singlet oxygen on protein damages in MR is demonstrated here by the treatment of β-carotene (Fig 7), which is a powerful singlet oxygen

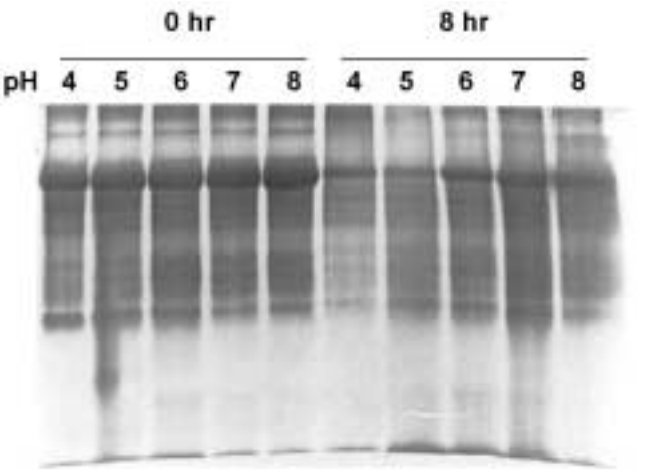

Fig. 6. Effect of pH on BSA protein damages caused by MR in presence of ferrous iron. Proteins were treated for 0 and 8 hr and analyzed by 15% SDS-PAGE and silver stained.

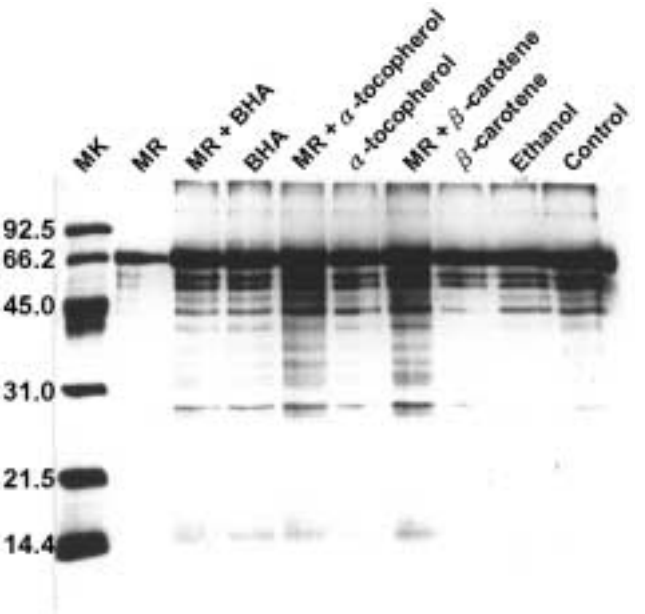

Fig. 7. The effects of antioxidants on BSA protein damage caused by MR. BSA was treated for 4 hr and protein structure was analyzed by 15% SDS-PAGE and silver stained. MK, protein molecular markers. Size is in kDa.

scavenger and significantly protects protein from degradation by MR. Other antioxidant compounds including α-tocopherol and BHA also showed protection on protein damages caused by MR. There is no doubt that ROS play an important role on protein damages by MR photodynamic action.

The damages of ROS on protein structure often include protein oxidation, degradation and cross-linking[10, 15, 16, 24]. The formation of larger polypeptides resulting from the cross-linkage of smaller size proteins in MR treatments

were shown in Figures 2 and 3. When red blood cells are exposed to ROS, cell membrane protein will form cross-links with reducible (with disulfide bond formation) and non-reducible bonds [10]. H_2O_2 promotes reducible protein cross-link formation while hydroxyl radicals are required for non-reducible cross-linking. In MR, due to the existence of hydrogen peroxide and hydroxyl radicals, protein cross-linkages might occur with both reducible and non-reducible bonds.

BSA, catalase, peroxidase and superoxide dismutase are biologically important antioxidant proteins, protecting biological cell from the damages of oxidants. All the four proteins were dramatically and rapidly damaged under MR photodynamic action either at the protein structure or the enzymatic activity levels. This study strongly suggests that the failure of cellular defense system mediated by antioxidant proteins is the key factor resulting in cell death by MR photodynamic action.

ACKNOWLEDGEMENT

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摘 要

李敏惠[1,2]、曾德賜[1]、許富翔[1]. 2006. 甲硫胺酸與核黃素之光動效應對抗氧化蛋白之影響. 植病會刊 15:17-24. ([1]台中市中興大學植物病理學系；[2]聯絡作者，電子郵件信箱：mhlee@nchu.edu.tw；傳眞：+886-4-2287-7585)

甲硫胺酸 (methionine) 與核黃素 (riboflavin) 之光動效應 (簡稱 MR) 可產生高量的活化態氧 (reactive oxygen species, ROS)，爲一致死能力極強之殺菌劑。本研究利用四種抗氧化蛋白，包括過氧化氫酶 (catalase)、過氧化酶 (peroxidase)、超氧岐化酶 (superoxidase dismutase, SOD) 及小牛血清蛋白 (bovine serum albumin, BSA)，以研究 MR 對細胞內重要抗氧化蛋白結構及酵素活性的影響，及探討 ROS 在 MR 對蛋白所產生之傷害所扮演的角色。本研究發現 MR 對蛋白質所造成之傷害包括結構裂解及蛋白分子交互鏈結成大分子蛋白。所有處理之蛋白皆可被 MR 裂解成小分子蛋白，且裂解情形隨著照光處理時間之增加而加重。經由蛋白分子交互鏈結而形成之大分子蛋白則可發生於經 MR 處理的 SOD 及 catalase。酵素活性包括 catalase、peroxidase 及 SOD 皆受到 MR 的破壞，此破壞情形於照光處理 15 分鐘後即明顯顯現。無論在蛋白結構的裂解或酵素活性的破壞，此四種受MR處理的蛋白分子中，以 SOD 對 MR 最爲不敏感。利用具有消除 ROS 能力或具有促進 ROS 產生能力的化合物進行研究，發現 ROS 爲 MR 對蛋白造成傷害之必需因子。在極短的處理時間 MR 即對細胞內重要的抗氧化蛋白產生破壞，此顯示細胞喪失抗氧化防禦機制可能爲 MR 造成細胞快速死亡重要原因。

關鍵詞：活化態氧、自由基、氧化氫酶、過氧化酶、超氧岐化酶、小牛血清蛋白、抗氧化蛋白、蛋白裂解、蛋白分子交互鏈結、細胞死亡