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Low-molecular Peptide Model of Protein Denaturation by UVA Irradiation and the Effect of Antioxidants

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Abstract

As a simple model of ultraviolet (UV)-induced protein denaturation, Amyloid P hexapeptide (APH, Phe-Thr-Leu-Cys-Phe-Arg) was exposed to UVA irradiation. A new product was isolated by HPLC and identified by fast atom bombardment mass spectrometry (FAB-MS) as the disulfide dimer. Thus, UVA induced disulfide bond formation. We also examined the effect of replacing the Cys residue of APH with other amino acid residues (methionine, tryptophan, tyrosine and histidine) that might be susceptible to oxidative damage. In the case of [Trp^4]-APH, the amount of the analog was decresed at 40 J/cm^2 or higher. When APH and [Trp^4]-APH were mixed and UVA-irradiated, the combination afforded a greater number of products than the sum of those obtained when they were separately irradiated. That finding suggests that denaturation of proteins by UVA may involve very complex reactions of plural kinds of amino acid residues. Since the UV-induced APH dimer formation could be easily evaluated by HPLC, we used this system to examine the effect of antioxidants on the UVA-induced reaction. The dimer formation was greatly inhibited by dithiothreitol and N-acetylcysteine, but was promoted by azide and catechin. This system might be useful for evaluating the ability of drugs to prevent oxidative damage to proteins caused by UVA irradiation.

Keywords: *protein denaturation, disulfide bond, oxidative damage, peptide model, ultraviolet radiation*

Introduction

Protein denaturation by ultraviolet (UV) radiation is considered to be one of the chief causes of immune response abnormalities (Ichikawa *et al.*, 1995) such as light senescence or chronic light hypersensitivity and photo-aging (Kato *et al.*, 1995). Therefore it is very important to understand its mechanism. There have been some observations suggesting

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that active oxygen may be linked to protein denaturation by UV (Mathews, and *Kinsky et al.*, 1987), and that aromatic amino acids, phenylalanine, tryptophan and tyrosine, may undergo radical-induced cross-linking when exposed to UV (Weadock *et al.*, 1995). Furthermore, it was reported that bityrosine was produced when bovine serum albumin was exposed to ^{60}Co radiation (Davies 1987). As a new approach to analyzing the complex mechanisms of protein denaturation by UV light, we decided to expose a low-molecular peptide to UVA irradiation and then to analyze the products formed by means of HPLC and fast atom bombardment mass spectrometry (FAB-MS). The criteria for the model peptide were as follows; 1) a relatively small molecular weight, for ease of HPLC separation and product structure analysis; 2) the presence of a cysteine residue, so that disulfide bond formation could be analyzed; 3) the absence of other amino acids likely to be oxidized by active oxygen, such as methionine, tryptophan, tyrosine and histidine residues (Davies *et al.*, 1987), to avoid over-complexity. Based on these criteria, we chose the amyloid P hexapeptide Phe-Thr-Leu-Cys-Phe-Arg (APH). We also examined peptides in which the Cys residue of APH was replaced with methionine, tryptophan, tyrosine or histidine. Because HPLC makes it easy to analyze products generated in this model system, we also examined the effects of various antioxidants on the UVA-induced dimer formation of APH.

Materials and Methods

Peptides

APH was purchased from Cosmo Bio Co., Ltd. After being dissolved in purified water, it was preserved at -20 ℃ until used. The amino acid sequence of APH is Phe-Thr-Leu-Cys-Phe-Arg (M.W. = 784.4). Peptides containing Met, Trp, Tyr or His in the fourth place of Cys in APH were synthesized at Kurabo Industries Ltd. These analogs are expressed [replaced amino acid such as Met, Trp, Tyr or His^4]-APH as a rule. Their purity of each peptides was 95 % or higher.

Effect of various drugs on APH dimer formation

All drugs used in the study were purchased from Sigma-Aldrich Co., Ltd. Catechin is a typical natural antioxidant that is present in tea (Nanjyo *et al.*, 1993). Dithiothreitol is well known to block disulfide dimer formation. N-acetylcysteine was reported to inhibit intracellular signaling initiated by UVA (Devary *et al.*, 1992). Azide was reported to block singlet oxygen formation by UVA irradiation (Wlaschek *et al.*, 1995). Glutathione, the most common thiol in the body, is a strong antioxidant (Nishikimi *et al.*, 1980).

Each drugs was mixed into an APH solution and the mixture was exposed to UVA at 160 J/cm^2. Final concentration of each drug was 1 mM.

UVA Irradiation

As a UVA light source, FL20S BLB fluorescent lamps were used (six in parallel; Toshiba Co., Ltd., Japan), which emit at 300 - 410 nm. The incident light was passed through a 2mm-thick ultraviolet absorbing glass filter (Longpass filter WG335, Schott, Germany) to eliminate wavelengths below 320 nm. Dose was measured with a UV radiometer UVR-3036/S (Topcon Co., Ltd., Japan). Each peptide (1 mg/ml) of 200 μl was put into a test tube, which was sealed with parafilm during irradiation.

Analysis by HPLC

We used an LC-10A HPLC system (Shimadzu Co., Ltd., Japan) with a Capcell Pak C18 (4.6× 250 mm, Shiseido Co., Ltd., Japan) reverse-phase column. A 20 μL peptide sample was injected, and eluted at 1 ml/min with a 15 % to 50% acetonitrile/0.1% trifluoroacetic acid (TFA) linear gradient over 35 min. The column temperature was 40 ℃, and the detection wave-

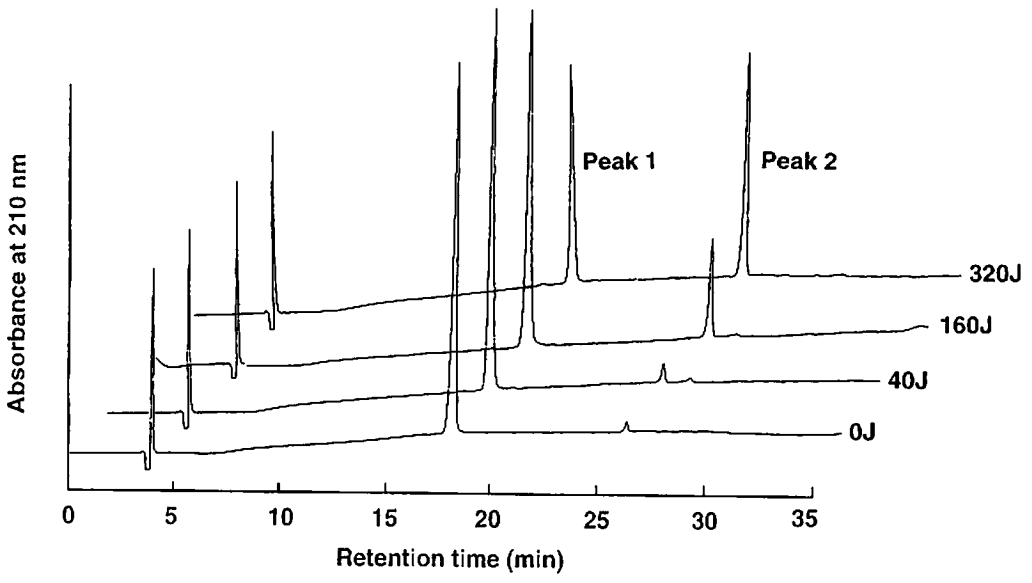

Fig. 1 Effect of UVA irradiation on APH
APH was dissolved in purified water at 1 mg /mL, then irradiated with UVA at various doses up to 320 J/cm^2. Each sample was analyzed by reverse-phase HPLC.

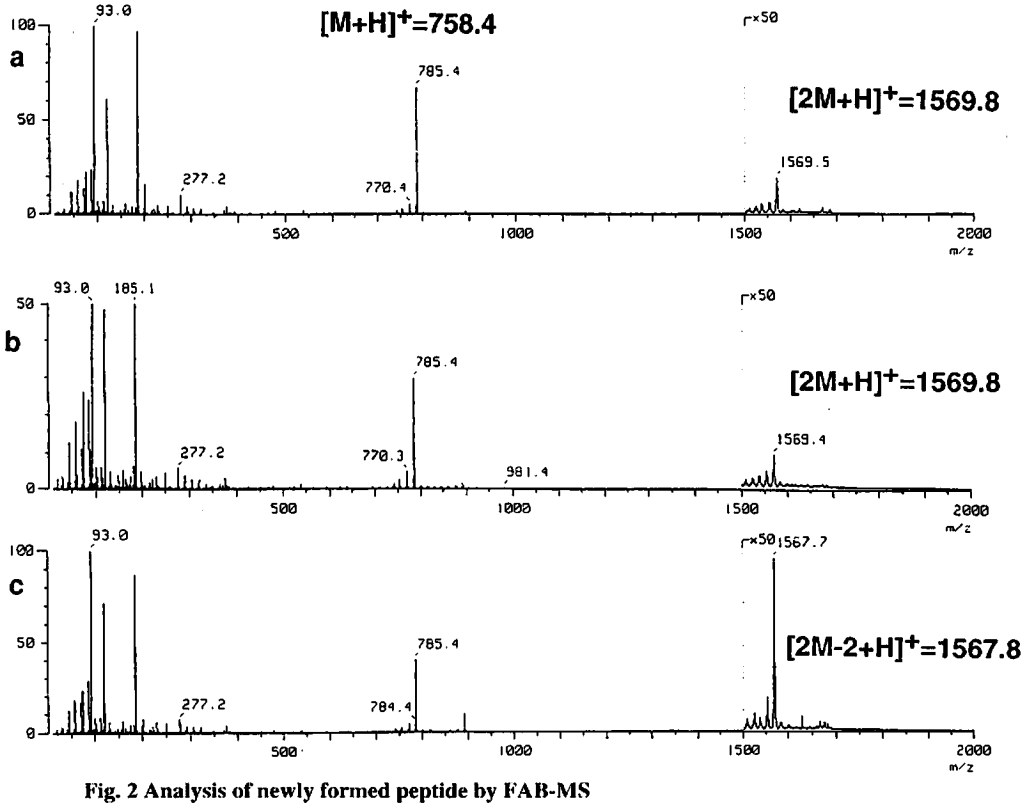

Fig. 2 Analysis of newly formed peptide by FAB-MS
a; APH (before irradiation), b; Peak 1 (after UVA 160 J/cm^2 irradiation), c; Peak 2 (after UVA 160 J/cm^2 irradiation).

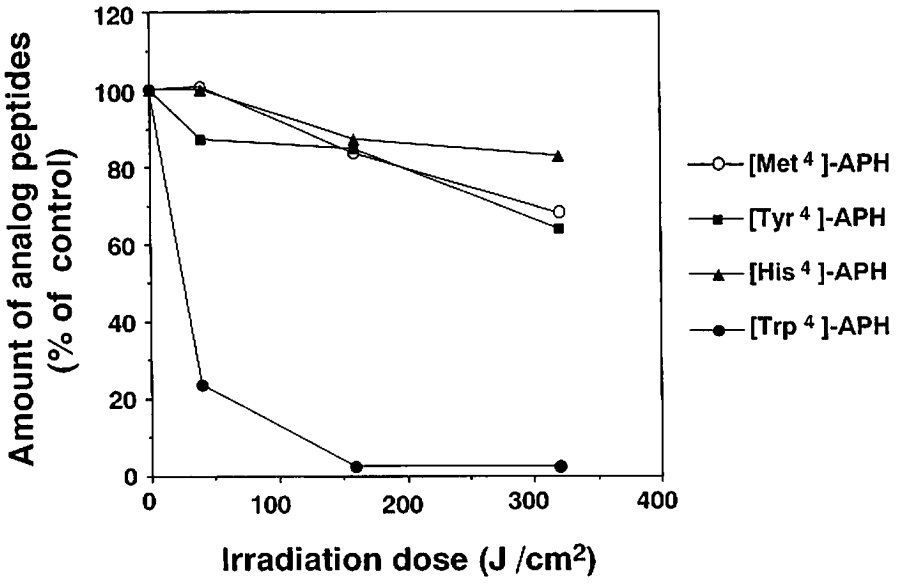

Fig. 3 Effect of UVA irradiation on APH analogs containing various amino acids in place of the Cys residue.

After irradiation with UVA at various dose, each APH analogs was analyzed by HPLC.
The amount of these alanogs was calculated from the resulting peak area as a % of control.

length was 210 nm.

Structural Analysis of Peptides

A new peptide isolated by HPLC after UVA irradiation of APH was structurally analyzed by using a two-sector mass spectrometer with a fast atom bombardment ion source (JMS SX-102A, JEOL Ltd., Japan). It was dissolved in 5 % acetonitrile. Xe gas was used for fast atom bombardment (FAB) ionization. Other conditions were as follows; gun voltage, 5 kV; emission current, 5 mA. The magnetic field scan rate was 0.05 S/decade, and the mass range was from m/z 50 to 2,000. The specimen was prepared by placing a 0.5-1 μl aliquot of test solution on the target, and after about one minute, 1 μl of glycerol/thioglycerol (1:1, v/v) matrix was added.

Results

Effect of UVA Irradiation on APH

As shown in Fig.1, the peak of APH (Peak 1) decreased concomitantly with the appearance of a new peak (Peak 2) in a UVA-dependent manner. Figure 2 shows the FAB-MS of Peak 1 and Peak 2. The spectrum of Peak 1 closely matched that of intact APH, indicating Peak 1 to be the unreacted peptide, as expected from its retention time. The ion at m/z 1569.5 is a gas-phase-generated dimer ion. On the other hand, in the spectrum of Peak 2, the m/z ion matched the calculated value of the protonated molecular ion of the disulfide-bonded dimer. The m/z 785.4 ion corresponds to the molecular weight of intact APH. However, since Peak 2 had been purified by HPLC, the ion could not be due to contaminating APH. Because FAB ionization is known to partly reduce S-S bonds (Larsen *et al.*, 1985), it is likely that the m/z 785.4 ion was an artifact (= monomer) generated by reduction during the FAB process. This explanation also supports the S-S homodimer structure predicted from the molecular weight. The other residues of APH did not appear to undergo any UVA-induced change.

Effect of UVA irradiation on APH analogs

Similar experiments were conducted using

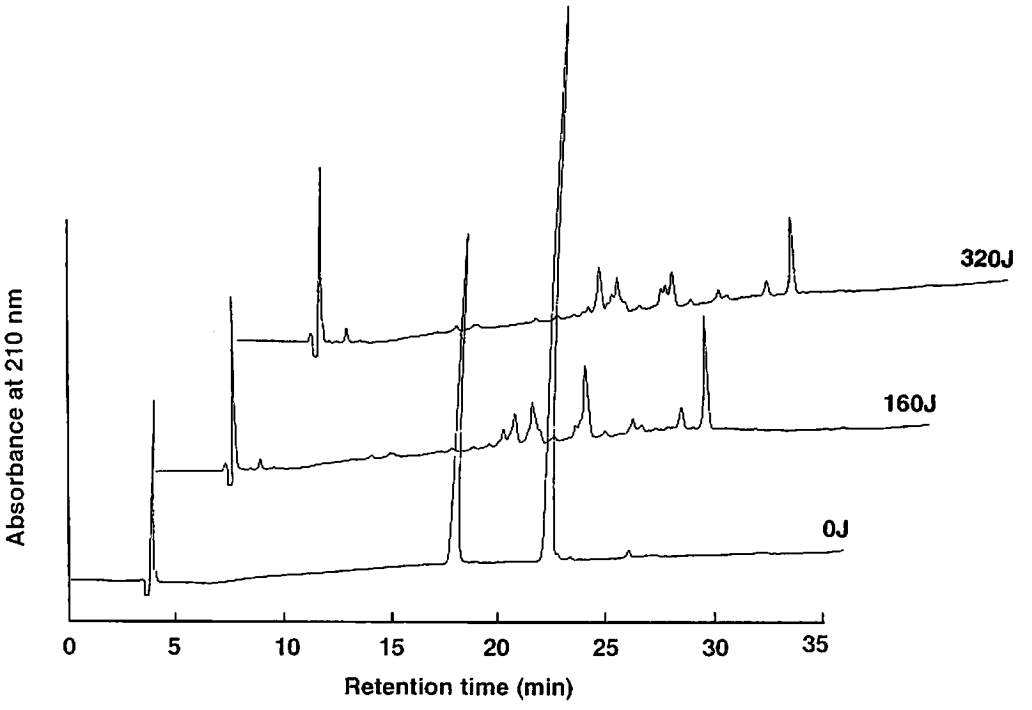

Fig. 4 Effect of UVA irradiation on a mixture of APH and [Trp^4]- APH.
APH and [Trp^4]- APH were mixed and irradiated at 160 J/cm^2 or 320 J/cm^2.
The retention time of APH was about 17 min and the peak behind the APH peak was that of [Trp^4]- APH.

APH analogs in which the Cys residue of APH was replaced with Met, Tyr, His or Trp. The results are shown in Fig. 3. In the case of [Met^4]-APH, [Tyr^4]-APH and [His^4]-APH, the amount of analogs slightly decreased in a UVA dependent manner. In the case of [Trp^4]-APH, little of the analog remained at 40 J/cm^2 or higher, while a number of small new peaks appeared (data not shown).

Effect of UVA Irradiation on a mixture of APH and [Trp^4]-APH

As indicated in Fig. 4, the mixture of APH and [Trp^4]-APH (additional amount of [Trp^4]-APH was equal to the amount of APH) generated more new peptides upon UVA irradiation at 160 J/cm^2 and 320 J/cm^2 as compared with the sum of those generated when the peptides were irradiated singly. Furthermore, the peak patterns were different for 160 J/cm^2 and 320 J/cm^2 irradiation. This suggests that additional reactions involving the Cys and Trp residues took place.

Effect of Drugs on APH Dimer Formation by UVA Irradiation

We examined the effect of various reducing agents or radical scavengers on disulfide dimer formation from APH under UVA irradiation. The results are shown in Fig. 5. Catechin promoted the dimer formation at 160 J/cm^2. Cysteine slightly inhibited the dimer formation. Dithiothreitol almost completely inhibited the dimer formation. N-acetylcysteine strongly inhibited the dimer formation. Azide facilitated dimer formation. Glutathione did inhibit the dimer formation. A new peak appeared under UVA irradiation in the presence of cysteine or glutathione (data not shown).

Discussion

We used APH as a model peptide to study protein denaturation by UVA because the

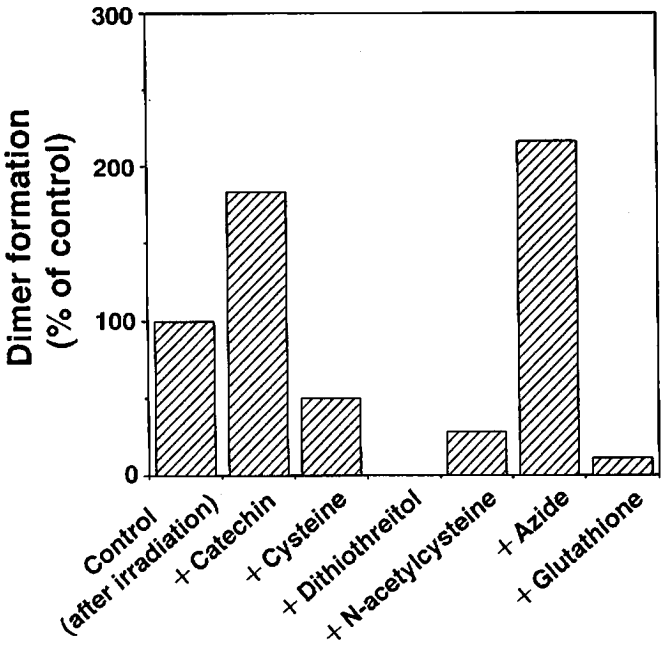

Fig. 5 The effect of various drugs on APH dimer formation by UVA irradiation.

APH was exposed to UVA at 160 J/cm^2 in the presence of various drugs. Final concentration of each drug was 1 mM. The amount of dimer was calculated from the resulting peak area as a % of control.

small number of residues should reduce the complexity of the reactions, and because the low-molecular products can be easily analyzed by reverse-phase HPLC, followed by FAB-MS. We found that UVA irradiation resulted in the formation of a disulfide-linked dimer of APH. There have been some reports suggesting an increase of disulfide bonds in proteins after UVA exposure. For example, the loss of free sulfhydryl groups can be accelerated in guinea-pig lens by *in vitro* UV exposure (Barron *et al.*, 1988), and the addition of low concentrations of cysteine inhibited protein denaturation induced by UV irradiation (Watanabe *et al.*, 1991). The other residues of APH, phenylalanine, threonine, leucine and arginine, appeared to be stable, at least up to 320 J/cm^2. In analogs of APH containing, methionine, tryptophan, tyrosine and histidine in place of the cysteine residue, UVA irradiation cause the decrease of each alalogs. These results suggest that amino acids such as methionine, tryptophan, tyrosine and histidine take part in protein denaturation by UVA. It has already been clarified that tyrosine dimer formation by the hydroxyl radical is involved in the polymerization of proteins under γ ray irradiation (Davies 1987). Since no such change was seen with [Tyr^4]-APH, γ rays and UVA may differ in their active oxygen-generating ability. It was reported that aggregation of collagen by UVA in the presence of riboflavin involves the formation of dityrosine (Kato *et al.*, 1994). Rivoflavin is known to generate active oxygen species when exposed to UVA. In living tissue, endogenous chromophores are thought to play an important role in denaturations of proteins. Further study may allow us to identify new mechanisms of protein denaturation by UVA irradiation.

Since proteins contain many kinds of amino acids, we also examined the effect of UVA on a mixture of APH and [Trp^4]-APH. The results showed that the number of new peaks that appeared was greater than the sum of those generated from the individual proteins. In

other words, further reactions may have occurred among the initially generated products. This is presumably one reason why it is so difficult to analyze the UV-induced denaturation of proteins.

We examined the effect on UVA-induced APH dimer formation of various drugs that might be expected to inhibit oxidative damage. Cystein, dithiothreitol and N-acetylcysteine inhibited the dimer formation. Since these drugs does not absorb UVA (Wetlaufer 1962; Devary *et al.*, 1992), these inhibitory effects were not due to UVA absorption of themselves. Cysteine and glutathione inhibited the dimer formation, but new peaks appeared. These may be due to disulfide heterodimer formation. Catechin and azide promoted oxidative damage, even though they have been reported to have antioxidant activity (Nanjyo *et al.*, 1993; Wlaschek *et al.*, 1995). These results suggest that catechin and azide can play a dual role as both radical scavengers and radical mediators, especially in the formation of disulfide bonds. This model system is expected to be useful for analysis of the mechanisms of protein degradation by UV exposure, and may allow the identification of new protective agents against phototoxicity or photoallergy.

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