Current Topics in Analytical Chemistry
Vol. 9, 2012

Original Communication

Analysis of protein damage based on the fluorometry of tryptophan residue

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ABSTRACT

Photosensitized protein damage is an important process in phototoxicity and the mechanism of photodynamic therapy. Fluorometry could be used for the evaluation of the protein photo-damaging activity of a photosensitizer. In this study, we designed a procedure for the analysis of protein damage by fluorometry using a water-soluble protein, human serum albumin (HSA). HSA has one tryptophan residue, which is a strongly fluorescent amino acid and is easily oxidized by photochemical reaction. The oxidation of HSA results in a decrease of the fluorescence intensity of the tryptophan residue. These properties are appropriate to probe oxidative protein damage. On the basis of these properties of HSA and tryptophan, an analytical procedure for protein damage is proposed.

KEYWORDS: protein damage, human serum albumin, tryptophan, fluorometry, photosensitizer

1. INTRODUCTION

Fluorometry, an analysis method based on fluorescence measurement, is a simple and highly sensitive method used in analytical chemistry [1-7]. It can be used in medicine to determine biomolecular damage. Photosensitized protein damage is an important mechanism in photodynamic therapy (PDT), which is a promising treatment for cancer and non-malignant conditions [8-10]. PDT involves the administration of a photosensitizer followed by exposure of the tissue to visible non-thermal light. When the photosensitizer is illuminated with light of appropriate wavelength, the photoexcited molecules induce photochemical damage to biomacromolecules, including protein, resulting in cell death. In general, important mechanisms of biomolecule damage are electron transfer (type I) [11, 12] and the generation of reactive oxygen species, such as singlet oxygen ($^1O_2$) (type II) [13-15]. A simple method to evaluate the damaging activity of photosensitizers against biomolecules is an important requirement in developing a PDT photosensitizer. In this paper, a simple method of the fluorometry of protein damage using human serum albumin (HSA) is introduced. Several examples of the fluorometry of HSA damage photosensitized by porphyrin derivatives are also presented.

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2. MATERIALS AND METHODS

2.1. Materials

HSA and tetrakis(1-methyl-4-pyridinio)porphyrin (H_2TMPyP) were from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). Sodium azide (NaN_3) was from Wako Chemicals Co. (Osaka, Japan). Deuterium oxide (D_2O) was from Acrross Organics (New Jersey, USA). Sodium phosphate buffer (pH 7.6) was from Nacalai Tesque (Kyoto, Japan). DiethoxyP(V)tetraphenylporphyrin (EtP)

and bis(2-trifluoroethyl)P(V)tetraphenylporphyrin (F_3EtP) were synthesized according to the literature [16].

2.2. Detection of photosensitized HSA damage

As a target protein, HSA, a water-soluble protein, was used. The sample solution containing photosensitizers and 10 μM HSA in a sodium phosphate buffer (pH 7.6) was irradiated with a light-emitting diode (LED) (λ_{max} = 519 nm, 1 mW cm^{-2}, CCS Inc., Kyoto, Japan). The intensity of the LED light source was measured with a light meter (LM-331, AS ONE, Osaka, Japan). Protein damage by photosensitizers was evaluated by measurement of the fluorescence intensity from the amino acid residues [17]. The excitation and detection wavelengths were 298 and 350 nm, respectively.

2.3. Calculations of absorption spectra

The absorption transitions of tryptophan and *N*-formylkynurenine were calculated by the semi-empirical Zerner's intermediate neglect of the differential overlap (ZINDO) procedure utilizing the CAChe WorkSystem Pro 6.0 (Fujitsu, Ltd., Tokyo, Japan). The geometry of these compounds was obtained by a semi-empirical calculation at the PM5 level utilizing the CAChe WorkSystem Pro 6.0.

3. RESULTS AND DISCUSSION

3.1. Absorption and fluorescence properties of HSA

HSA has an absorption band around 200 ~ 300 nm, which is assigned to the π-π transition of aromatic amino acid residues (Figure 1). The tryptophan residue has the longest absorption band around 270 ~ 300 nm in the amino acid residues of HSA. Because tryptophan is easily oxidized, tryptophan oxidation can be used as the probe for HSA damage. On 298 nm excitation, tryptophan residue can be selectively excited. The tryptophan residue demonstrates fluorescence around 350 nm (Figure 2). The concentration of the tryptophan of HSA can be estimated from the relative intensity of the fluorescence at 350 nm on the 298 nm excitation.

In general, photosensitized protein damage is explained by two mechanisms, type I and II

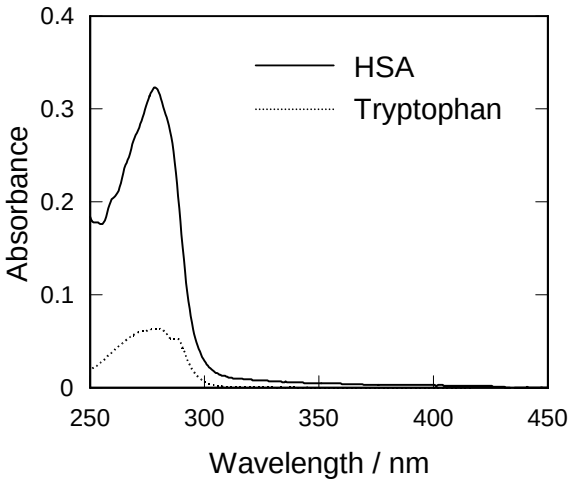

Figure 1. Absorption spectra of 10 μM HSA and tryptophan in a 10 mM sodium phosphate buffer (pH 7.6).

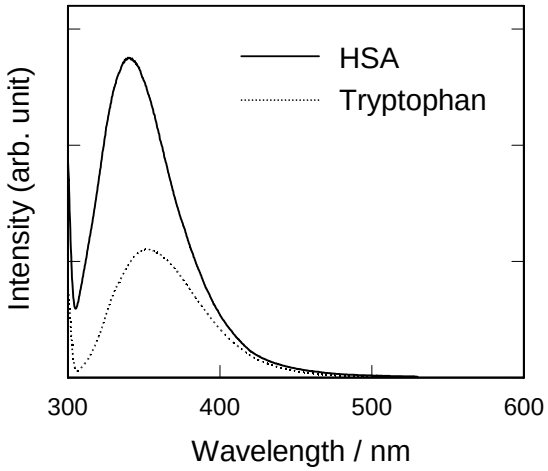

Figure 2. Fluorescence spectra of 10 μM HSA and tryptophan in a 10 mM sodium phosphate buffer (pH 7.6). Ex: 298 nm.

(mainly $^1O_2$ generation). With the type I mechanism, a radical cation of tryptophan is formed through electron transfer. The radical cation undergoes a reaction with the surrounding elements, such as molecular oxygen or water [18]. The important final product is *N*-formylkynurenine (Figure 3). Since *N*-formylkynurenine is not easily obtained, its absorption spectrum was investigated by calculation. This product cannot absorb the wavelength around 298 nm (Figure 4). Furthermore, fluorescence is not observed around 350 nm [18].

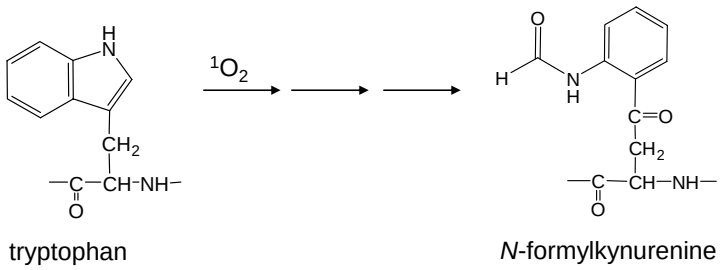

Figure 3. Scheme of the HSA oxidation and formation of *N*-formylkynurenine.

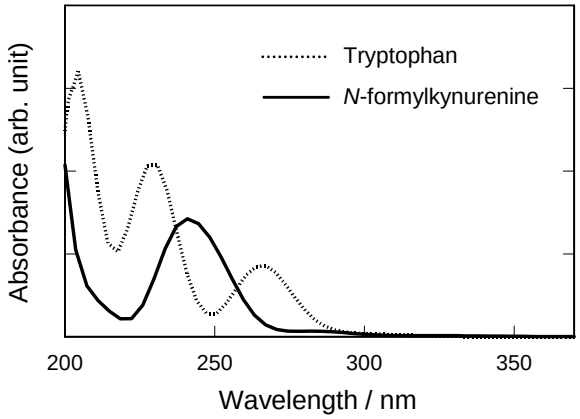

Figure 4. Calculated absorption spectra of tryptophan and *N*-formylkynurenine.

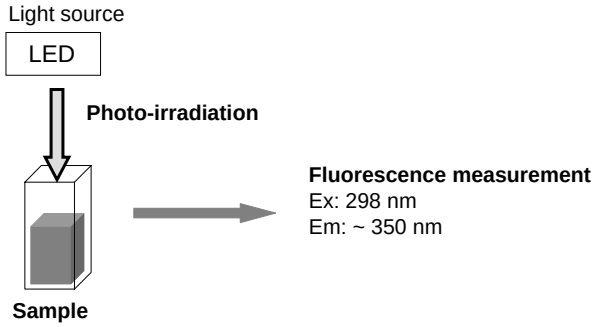

Figure 5. Scheme of the fluorometry of protein photodamage.

Therefore, the fluorescence intensity at around 350 nm of the tryptophan residue on the 298 nm excitation decreases depending on the extent of protein damage.

3.2. Protocol of the fluorometry of the HSA photo-oxidation

The phenomenon reported above can be applied to the determination of the protein-damaging activity of visible-light photosensitizers. The simple scheme for this fluorometry is presented in Figure 5. For the fluorometry of the oxidized tryptophan of HSA via a photosensitized reaction, a sample solution containing 10 μM HSA and photosensitizers are mixed in an appropriate buffer, such as a 10 mM sodium phosphate buffer (pH 7.6). The appropriate absorbance of the photosensitizer at the maximum wavelength of the light source is about 0.01 ~ 0.5. The concentration of the photosensitizers should be adjusted to this condition. An LED is recommended as the light source because of its defined emission spectrum and reduced heat formation. The sensitivity of this assay is proportional to the irradiation time. The quantum yield of tryptophan oxidation (Φ_{ox}) can be determined by the measurement of the fluorescence intensity and the molar quantity of absorbed photon by the photosensitizer (*AP*, mol). The values of *AP* can be calculated by the following equation:

$$AP = \int \left(1 - 10^{-Abs(\nu)}\right) E(\nu) S d\nu \times t \qquad (1)$$

where *Abs(ν)* represents the absorbance of the photosensitizer, $E(\nu)$, the photon fluence of the light source at the indicated light frequency (mol cm^{-2} min^{-1} s), ν, the light frequency (s^{-1}), *S*, the irradiated area (cm^2), and *t*, the irradiation interval [7]. The *AP* value can be estimated, in practice, with the following equation:

$$AP = \sum \left\{ \left(1 - 10^{-Abs(\nu)}\right) E(\nu) S \Delta\nu \right\} \times t \qquad (2)$$

where $\Delta\nu$ represents the sampling interval of the light frequency. The value of Φ_{ox} is calculated by the following equation:

$$\Phi_{ox} = \frac{\Delta I \times [\mathrm{HSA}] \times V}{I_0 \times AP} \quad (3)$$

where ΔI is the decreased fluorescence intensity, [HSA], the initial concentration of HSA (10 μM), V, the volume of the sample, and I_0, the initial fluorescence intensity of HSA.

The contribution of the mechanisms, type I and II, can be estimated from the effect of NaN_3, which is a typical quencher of $^1O_2$ [19]. Although NaN_3 can quench the photoexcited state of the photosensitizer, in general, $^1O_2$ is selectively quenched by NaN_3 during the relatively long lifetime of $^1O_2$. The examples for the determination of these two mechanisms are described in the following sections.

3.3. Photo-oxidation of HSA by cationic water-soluble porphyrin

Several examples of the fluorometry of HSA damage are presented in this section. H_2TMPyP (Figure 6) is a water-soluble cationic porphyrin photosensitizer [20, 21]. This porphyrin has been used as a photosensitizer model for damage to biomolecules, such as to DNA [21]. The fluorescence intensity of the tryptophan residue of HSA decreased in a dose-dependent manner with photo-irradiation in the presence of H_2TMPyP, indicating the tryptophan photodamage by H_2TMPyP (Figure 7). The fluorescence decrement was partially inhibited by the addition of NaN_3, suggesting the contribution of $^1O_2$. However, HSA damage was not completely inhibited by an excess amount of NaN_3 (~10 mM). Because almost all $^1O_2$ can be quenched by 10 mM NaN_3, the damage of HSA photosensitized by H_2TMPyP with 10 mM NaN_3 should be due to the type I mechanism. These results suggest that the type I mechanism is partly responsible for HSA photodamage, as is the type II mechanism. The residual fluorescence decrement should be due to the type I mechanism. The extent of the inhibition of HSA damage by 10 mM NaN_3 was 0.83, suggesting that the contributions of type I and II are 0.17 and 0.83, respectively.

3.4. Photo-oxidation of HSA by phosphorus(V)porphyrin

In the next case, P(V)porphyrin derivatives (Figure 8) were used as the photosensitizer model

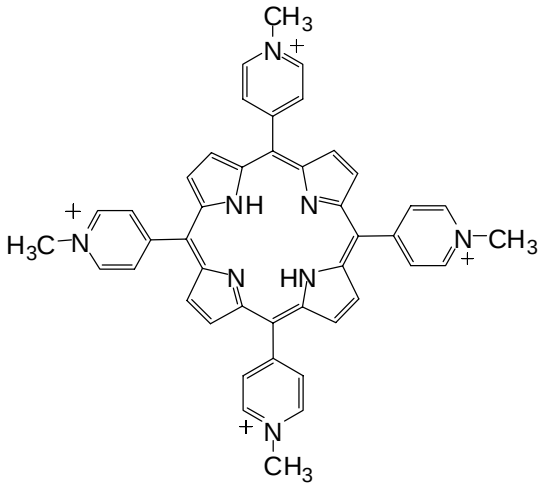

Figure 6. Structure of H_2TMPyP.

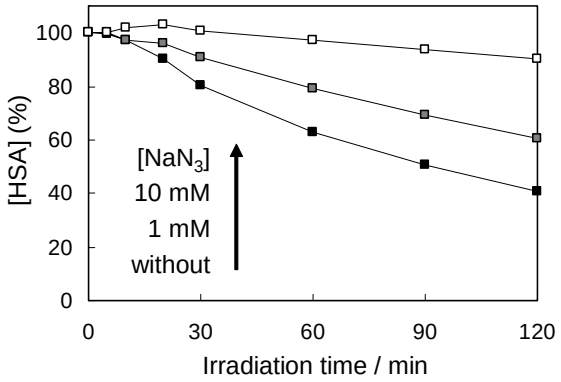

Figure 7. Effect of NaN_3 on HSA photodamage by H_2TMPyP. The sample solution contained 10 μM H_2TMPyP, 10 μM HSA, and the indicated concentration of NaN_3 in a 10 mM sodium phosphate buffer (pH 7.6). The vertical axis "[HSA]" indicates the relative concentration of non-damaged HSA.

[16]. P(V)porphyrins are water-soluble and have relatively strong photo-oxidizing activity [22]. In this study, two P(V)porphyrins, EtP and its fluorinated derivative, F_3EtP, were used to examine the effect of fluorination on the photosensitized protein-damaging activity of P(V)porphyrin. The intensity of HSA fluorescence around 350 nm, assigned to the tryptophan residue, was decreased by photo-irradiation in the presence of these P(V)porphyrins, similarly to the case of H_2TMPyP. The observed extent of this HSA damage by the fluorinated P(V)porphyrin, F_3EtP, was almost the same as that of the EtP (Figure 9). The Φ_{ox} of

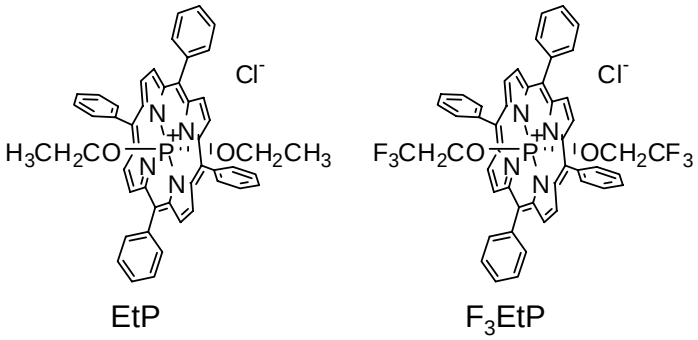

Figure 8. Structures of phosphorus(V)porphyrins.

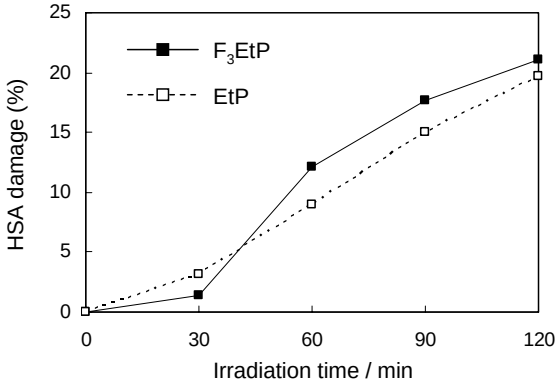

Figure 9. Time course of HSA damage photosensitized by F_3EtP and EtP. The sample solution contained 8 μM P(V)porphyrins and 10 μM HSA in a 10 mM sodium phosphate buffer (pH 7.6). The vertical axis "HSA damage" indicates the ratio of the damaged HSA, which is estimated from the ratio of the decreased fluorescence intensity.

tryptophan degradation photosensitized by P(V)porphyrins for 120 min irradiation was estimated from the decrease of the tryptophan fluorescence and the *AP* by the porphyrins using equations (2) and (3). The estimated yields were 2.9×10^{-5} and 2.2×10^{-5} for F_3EtP and EtP, respectively. The quantum yield of HSA photodamage by F_3EtP was slightly larger than that of EtP. This HSA damage was partially inhibited by NaN_3, similarly to the case of H_2TMPyP. Furthermore, HSA damage was enhanced in D_2O, in which the lifetime of $^1O_2$ is markedly elongated (about 2 ~ 4 μs in H_2O to 70 μs in D_2O) [23]. These findings suggest that the $^1O_2$ generation contributes to the HSA oxidation photosensitized by P(V)porphyrins. Similarly to the case of H_2TMPyP, HSA damage was not completely inhibited by an excess amount of NaN_3. The extents of the inhibition of HSA damage by 10 mM NaN_3 were 0.48 and 0.34 for F_3EtP and EtP, respectively. Consequently, the contribution of the type I and II mechanisms are summarized in Table 1. These results suggest that fluorination slightly enhances the protein photodamage by P(V)porphyrins.

Table 1. Contribution of the type I and II mechanisms to HSA photodamage by P(V)porphyrins.

Porphyrin	Type I mechanism	Type II mechanism	Φ_{ox}
F_3EtP	0.52	0.48	2.9×10^{-5}
EtP	0.66	0.34	2.2×10^{-5}

CONCLUSION

Tryptophan residue of HSA is easily oxidized through a photosensitized reaction. The extent of tryptophan damage could be estimated from the decrement of the fluorescence. These fluorescence reductions should depend on the extent of protein damage. Using this method, a simple fluorometry of protein damage was proposed. Water-soluble porphyrins, H_2TMPyP, and P(V)porphyrins effectively damaged HSA with photo-irradiation. The contribution of the type I and II mechanisms to photosensitized HSA damage by these porphyrins could be determined.

ACKNOWLEDGMENT

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education,

Culture, Sports, Science, and Technology (MEXT) of the Japanese Government (Grant Number 23750186).

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