Trends in Photochemistry & Photobiology
Vol. 16, 2014

Original Communication

Amino acids photocatalyzed by titanium dioxide can produce secondary hydrogen peroxide

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ABSTRACT

The effect of amino acids on the photocatalytic toxicity of titanium dioxide (TiO_2) was investigated. Various amino acids were treated with ultraviolet irradiation along with rutile and anatase types of TiO_2 particles. The photooxidized amino acids, especially cysteine, generated hydrogen peroxide in the presence of copper ions during the auto-oxidation process. In the case of aromatic amino acids, hydrogen peroxide formation from photocatalyzed phenylalanine was significantly larger than that from tyrosine. Hydrogen peroxide is a long-lived reactive oxygen species. Furthermore, photocatalyzed amino acids gradually generate hydrogen peroxide. This secondary generation of hydrogen peroxide may play an important role in the cytotoxicity of the TiO_2 photocatalyst after photoirradiation.

KEYWORDS: titanium dioxide, photocatalyst, amino acids, hydrogen peroxide, secondary reactive oxygen species

1. INTRODUCTION

The photochemistry of titanium dioxide (TiO_2) has been extensively studied [1-5]. In fact, photocatalytic reaction is one of the most important applications of TiO_2. The crystalline form of TiO_2 is a semiconductor, and the two crystalline forms, anatase and rutile, are well known. Photoirradiation applied to a TiO_2 crystal induces the formation of an excited electron in the conduction band and a hole (h^+) in the valence band, leading to the redox reaction of materials adsorbing on the TiO_2 surface, including water and/or molecular oxygen. The photocatalytic reactions together with the surface water and oxygen cause the formation of various reactive oxygen species (ROS) such as free- or trapped-hydroxyl radicals ($OH^\bullet$), superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) [2-5]. In the field of photobiology, TiO_2 is an important material because of its sunscreen [6] and phototoxic effects [2-5]. Indeed, the photocatalytic bactericidal activity of TiO_2 has been successfully applied. In addition, its photodynamic effect on cancer cells has been investigated [2-4, 7-10]. The administered small particles of TiO_2 in cancer cells induce cytotoxicity under ultraviolet A (UVA) irradiation. However, the phototoxic mechanism of TiO_2 has not been well understood. For example, although TiO_2 particles rarely incorporate into the cell nucleus [7], cellular DNA damage by TiO_2 photocatalysis has been reported [11-13]. We have previously reported the role of H_2O_2 in photocatalytic DNA damage [14]. H_2O_2 is a long-lived ROS and can penetrate into the nucleus membrane. In the presence of a metal ion such as iron or copper, more reactive ROS, i.e. $OH^\bullet$ and a copper-peroxo complex, can be formed. Recently, we reported the effect of the secondary generation of ROS from the photocatalyzed sugars on DNA photodamage by TiO_2 [15]. Oxidized sugars produce H_2O_2 during the auto-oxidation process and cause DNA damage. In cell, other ubiquitous biomolecules

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such as amino acids, especially aromatic amino acids, may be a secondary source of H_2O_2 in the TiO_2 photocatalytic reaction. In this study, the effect of amino acids on the phototoxicity of TiO_2 was investigated. Various amino acids (Figure 1) were treated with UVA irradiation along with rutile and anatase types of TiO_2 particles.

2. MATERIALS AND METHODS

TiO_2 particles (anatase and rutile, average diameter: 50-100 nm) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Catalase (45,000 units/mg from bovine liver) was from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). Copper(II) chloride ($CuCl_2$) dihydrate, ethanol, folic acid, 1,2-benzenediol, and 1,4-benzenediol were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-Amino acids, alanine, arginine, asparagine, cysteine, cystine, glutamine, glycine, histidine, phenylalanine, proline, serine, tryptophan, tyrosine, and histidine, were also purchased from Wako Pure Chemical Industries, Ltd.

The concentration of H_2O_2 generated from the oxidized amino acids was measured by fluorometry using folic acid according to the literature [16]. The sample solution for examining secondary H_2O_2 generation contained a 100 μg mL^{-1} TiO_2 dispersion with or without 0.1-1 mM amino acids in 500 μL of a 10 mM sodium phosphate buffer (pH 7.6). The solutions were exposed to UVA light using a LUV-16 16 W UV lamp (λ_{max} = 365 nm, 1 mW cm^{-2}, AS ONE, Osaka, Japan). The intensity of the UVA light source was measured with a UVX radiometer and UVX-36 sensor (UVP LLC, Upland, CA, USA). The TiO_2 particles were removed by centrifugation after the photocatalytic reaction. The reaction mixture containing 10 μM folic acid, 20 μM $CuCl_2$, and 100 μM treated amino acids in 1.2 mL of a 10 mM sodium phosphate buffer

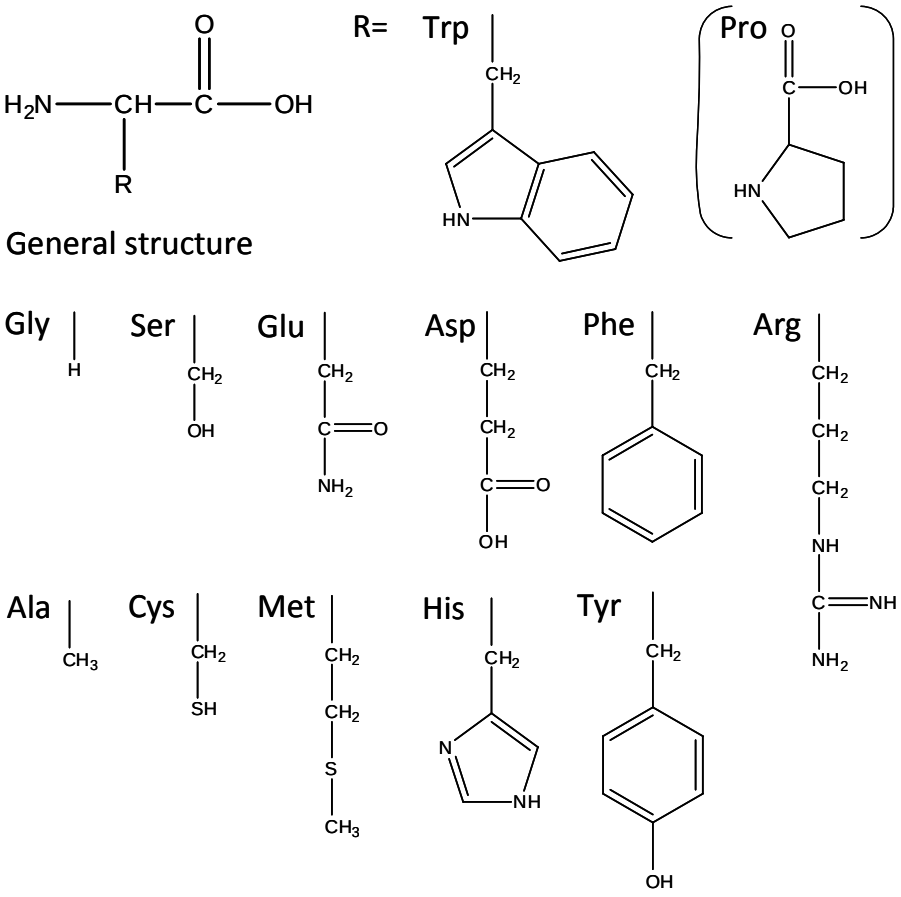


Figure 1. Amino acids used in this study.

(pH 7.6) was incubated in a 1.5 mL microtube (60 min, 37 ºC). The fluorescence spectrum of this solution was measured with an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The excitation wavelength was 350 nm. The fluorescence intensity was measured with a fluorescence photometer (650-60, Hitachi). The H_2O_2 formation rate was determined by similar fluorometry, according to the previous report [16].

3. RESULTS AND DISCUSSION

Photocatalyzed phenylalanine and tyrosine induced the formation of H_2O_2 in the presence of copper(II) ions (Figure 2). The sample solutions containing these amino acids (1 mM) and a 100 μg mL^{-1} anatase were irradiated with UVA light. The treated amino acids and folic acid were incubated in the presence of a copper(II) ion. The generated H_2O_2 was measured by fluorometry based on the oxidative decomposition of folic acid [16]. The concentration of generated H_2O_2 was determined by the calibration curve method and is presented in Figure 2. Because untreated phenylalanine and tyrosine could not generate H_2O_2 (data not shown), this H_2O_2 generation could be explained by secondary formation from the oxidized amino acids through their auto-oxidation mediated by the copper(II) ions. The TiO_2 photocatalytic reaction causes hydroxylation of aromatic compounds [17]. In such cases, the formation of benzenediol derivatives from the amino acids can be expected (Schemes 1 and 2). Indeed, benzenediol can generate H_2O_2 through the auto-oxidation (Scheme 3) [18, 19]. This auto-oxidation is markedly enhanced by metal ions such as copper(II) ion [19]. The amount of H_2O_2 generated from the photocatalyzed phenylalanine was significantly larger than that from tyrosine. This difference can be explained by the auto-oxidation rate of the corresponding oxidized products. The auto-oxidation of 1,4-benzendiol to 1,4-benzoquinone is markedly faster than that of 1,2-benzendiol (Scheme 3) [19]. It was confirmed that H_2O_2 generation from 1,4-benzendiol in a similar experimental condition was significantly larger than that of 1,2-benzenediol (Figure 3). Phenylalanine can be converted into various types of benzenediol, including a 1,4-benzenediol derivative (Scheme 1), whereas tyrosine cannot form the 1,4 type (Scheme 2). Therefore, phenylalanine might be an important source of secondary H_2O_2 in the TiO_2 photocatalytic reaction and the resulting cytotoxicity.

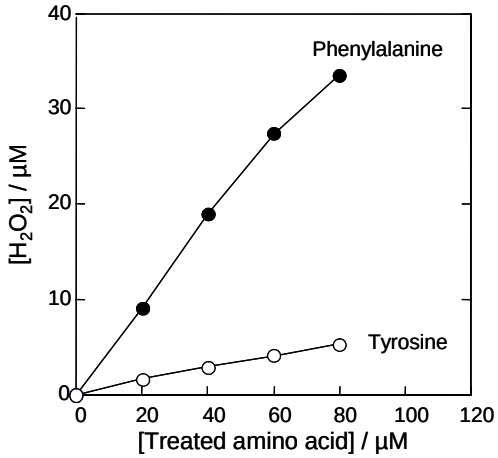
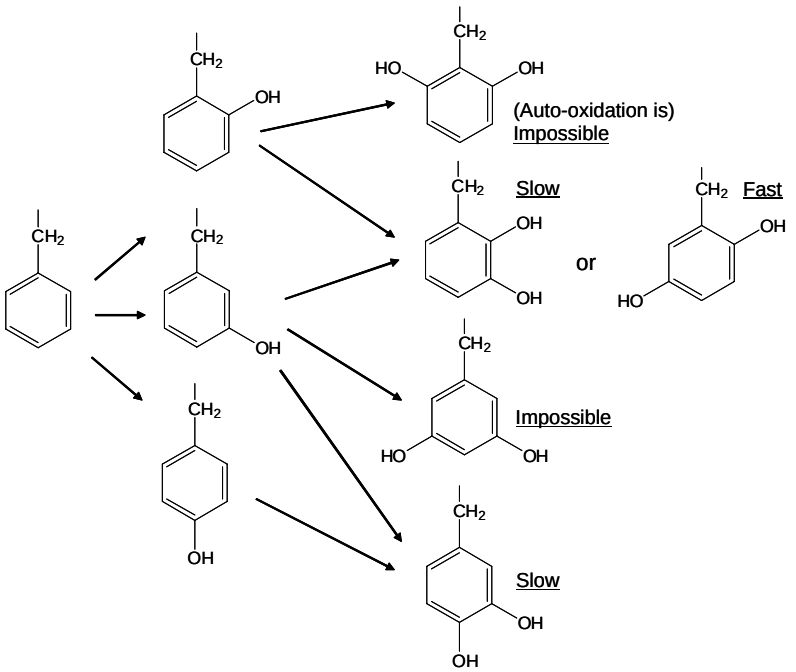
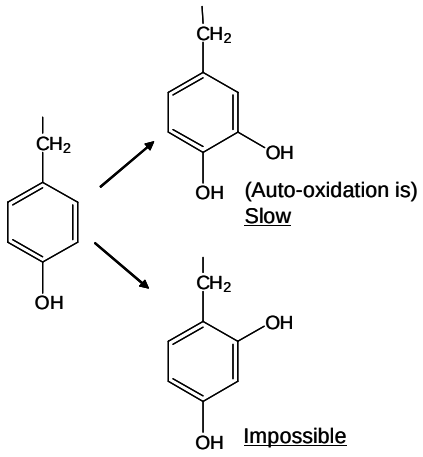


Figure 2. Secondary H_2O_2 formation from TiO_2-photooxidized phenylalanine and tyrosine. The 0.5 mL reaction mixture containing 1 mM phenylalanine or tyrosine with a 100 μg mL^{-1} anatase in a 10 mL sodium phosphate buffer (pH 7.6) was irradiated (λ_{max} = 365 nm, 6 J cm^{-2}). After photocatalytic reaction, the TiO_2 particles were removed by centrifugation, and the solution containing the oxidized amino acids was used. 1.2 mL of the sample solution containing the treated phenylalanine or tyrosine, 10 μM folic acid, and 20 μM $CuCl_2$ in a 10 mM sodium phosphate buffer (pH 7.6) was incubated (60 min, 37 °C), and the fluorescence intensity was measured (excitation: 360 nm, detection: 450 nm). The concentration of the generated H_2O_2 was determined by the calibration curve method.

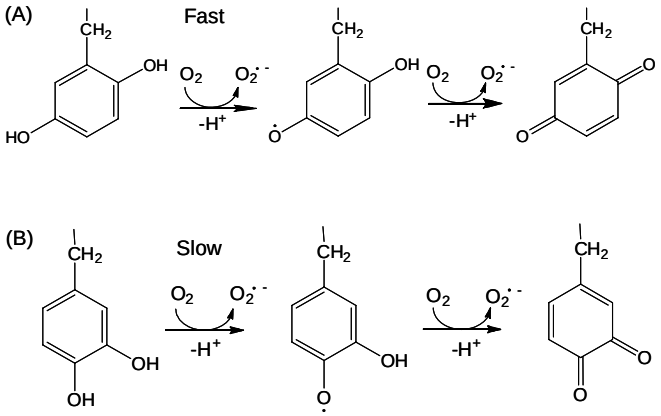
Thus, we used various amino acids to investigate the possibility of secondary generation of H_2O_2 in the TiO_2 photocatalytic reaction (Figure 4). In general, anatase induced a larger amount of secondary H_2O_2. It is generally accepted that the photocatalytic activity of anatase is larger than that of rutile. Especially, cysteine generated a large amount of secondary H_2O_2. The proposed mechanism of the H_2O_2 generation from photocatalyzed cysteine is shown in Scheme 4. Two cysteine molecules form cystine, a dimer of cysteine, through oxidation of the mercapto group. The reaction of cystine formation is easy at a higher pH condition. The secondary H_2O_2 formation depends on the pH, and the generation decreased in the higher pH condition (Figure 5). Since cystine did not generate H_2O_2 in a similar condition (data not shown), cysteine becomes an important source of the secondary



Scheme 1. Possible hydroxylation process of an aromatic ring of phenylalanine.



Scheme 2. Possible hydroxylation process of an aromatic ring of tyrosine.



Scheme 3. Possible auto-oxidation processes of 1,4- (A) and 1,2-benzenediol (B) compounds.

H_2O_2 formation in a neutral or acidic condition. In the case of phenylalanine, no significant pH effect was observed (Figure 5).

TiO_2 photocatalyzes the decomposition of water, resulting in $OH^{\bullet}$ formation, which is a strong oxidant. Alternatively, the formed hole in the valence band of TiO_2 can oxidize various compounds adsorbed on the TiO_2 surface. These reactive species can oxidize amino acids, leading to the secondary formation of H_2O_2 even under dark conditions (Figure 6).

Although TiO_2 is not likely to be incorporated into a cell nucleus [7], the generated H_2O_2 can be easily diffused and incorporated into a cell nucleus. Indeed, cellular [11-13, 20] and isolated [14, 21] DNA damage by TiO_2 photocatalytic reactions

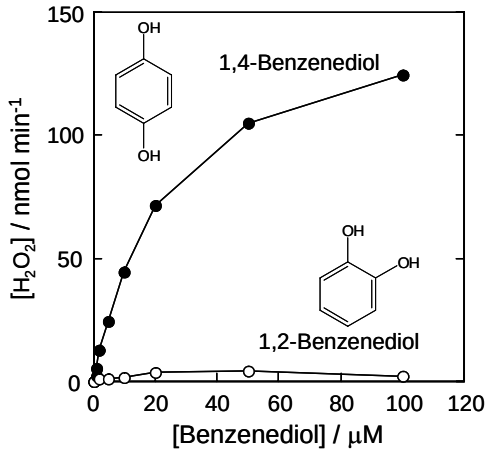


Figure 3. Hydrogen peroxide formation rate during the auto-oxidation of 1,2- and 1,4-benzenediol. 1.2 mL of the sample solution containing the indicated concentration of 1,2- or 1,4-benzenediol, 10 μM of folic acid, and 20 μM $CuCl_2$ was incubated (60 min, 37 °C). H_2O_2 formation rate was determined by the method described in the 'Materials and Methods' section.

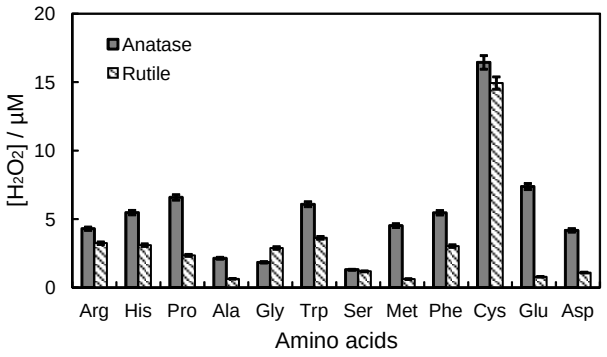
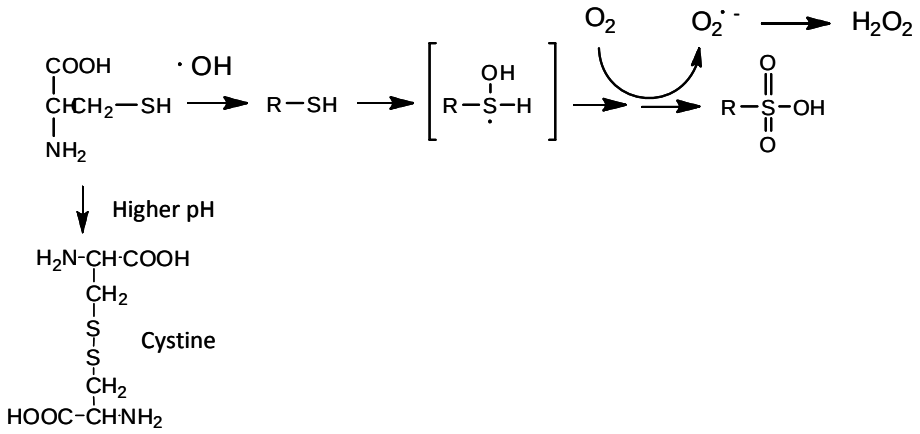


Figure 4. Secondary H_2O_2 formation from the TiO_2-photooxidized amino acids. 0.5 mL reaction mixture containing 100 μM amino acids with a 100 μg mL^{-1} anatase or rutile in a 10 mL sodium phosphate buffer (pH 7.6) was irradiated (λ_{max} = 365 nm, 6 J cm^{-2}). After photocatalytic reaction, the TiO_2 particles were removed by centrifugation, and the solution containing the oxidized amino acids was used. 1.2 mL of the sample solution containing 10 μM of treated amino acids, 10 μM folic acid, and 20 μM $CuCl_2$ in a 10 mM sodium phosphate buffer (pH 7.6) was incubated (60 min, 37 °C). The concentration of the generated H_2O_2 was determined by the same method described in the caption of Figure 2.



Scheme 4. Proposed mechanism of secondary H_2O_2 formation from the TiO_2-photooxidized cysteine and its oxidative dimerization.

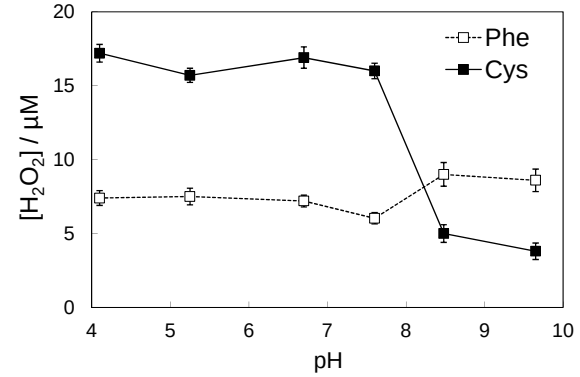


Figure 5. Effect of pH on secondary H_2O_2 formation from the TiO_2-photooxidized cysteine and phenylalanine. The 0.5 mL reaction mixture containing 100 μM cysteine or phenylalanine with a 100 μg mL^{-1} anatase in the sodium phosphate buffer (pH 4.1 – 9.7) was irradiated (λ_{max} = 365 nm, 6 J cm^{-2}). After photocatalytic reaction, the TiO_2 particles were removed by centrifugation, and the solution containing the oxidized amino acids was used. The 1.2 mL sample solution containing 10 μM of treated cysteine or phenylalanine, 10 μM folic acid, and 20 μM $CuCl_2$ in a 10 mM sodium phosphate buffer (pH 7.6) was incubated (60 min, 37 °C). The concentration of the generated H_2O_2 was determined by the same method described in the caption of Figure 2.

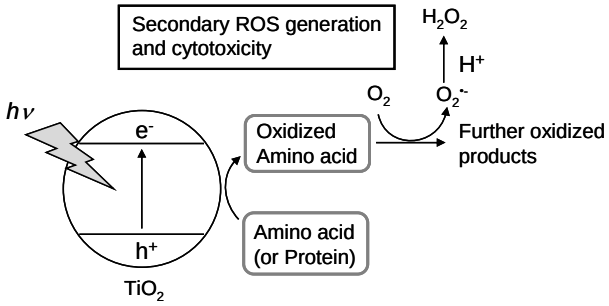


Figure 6. Proposed mechanism of secondary H_2O_2 formation from the TiO_2-photooxidized amino acids.

have been reported. Specifically, the secondary generation of H_2O_2 would require relatively long periods in the cell after photoirradiation. Secondary generation of H_2O_2 and the resulting DNA damage have also been reported [15]. *In vivo*, the cell membrane is an important reaction field for the TiO_2 photocatalyst because TiO_2 particles show affinity to cell membranes [7, 22]. The generated hole and $OH^{\bullet}$ can oxidize membrane proteins, leading to the formation of various oxidized amino acids. Oxidized cysteine and benzenediol derivatives from phenylalanine residue might play an important role in the dark cytotoxicity of the photocatalytic reaction of TiO_2 through secondary H_2O_2 generation.

CONCLUSIONS

In summary, the secondary formation of H_2O_2 from an oxidized amino acid by the TiO_2 photocatalytic reaction was demonstrated. In general, anatase was more active in the secondary H_2O_2 formation than rutile. The formation of H_2O_2 from the photocatalyzed cysteine was largest in the case of amino acids used in this study. In the case of aromatic amino acids, the H_2O_2 formation from photocatalyzed phenylalanine was significantly larger than in the case of tyrosine. This difference can be explained by the formation of a 1,4-benzenediol derivative from phenylalanine, the auto-oxidation of which is faster than that of other benzenediols. These findings suggest that the secondary H_2O_2 generation from cellular amino acids and decomposed proteins contributes to the photocytotoxicity of TiO_2 in addition to the direct formation of ROS by TiO_2 photocatalysis.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (417) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of the Japanese Government.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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