Effect of Hydrophobic Chain Length on the Antioxidation Properties of Alanyl Tyrosine Dipeptide-type Surfactants

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Abstract: The antioxidant (AOX) activities of alanyl tyrosine dipeptide-type surfactants with several chain lengths were investigated. The critical micelle concentration decreased exponentially with the carbon number of the hydrophobic chain of the surfactant. The antioxidative property was investigated using the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid ammonium salt) (ABTS) assay and the oxygen radical absorbance capacity (ORAC) assay. The AOX activity was found to be strongly dependent on the chain length in the monomer solution. Therefore, an increase in the molecular size negatively influenced the AOX ability of the tyrosine residue. However, it was almost independent of the chain length of the surfactant in the micellar solution. The micelle particles acted as a catalyst for the reduction of the radicals in the ORAC assay.

Key words: amino acid-type surfactants, antioxidative activity, micellar effect

1 Introduction

In the food and pharmaceutical industries, antioxidants (AOXs) have long been widely used[1-4], because the human body is always under the threat of oxidative stress. Free radicals are not only supplied by exogenous sources such as pollutants and photo radiation, but are also produced by the respiratory processes of living organisms. This strain competes with the AOX defenses of the body by enzymatic and non-enzymatic approaches, such as through vitamins. Failure of these processes leads to cancers, lifestyle diseases, aging, and the development of age-related diseases[5]. AOXs can delay or even retard oxidation in food or reduce oxidative stress in the cell mitochondria by capturing the free radicals associated with the chain reaction, resulting in the termination of propagation steps[6]. AOX enzymes, such as catalase and superoxide diastase, play an important role in the removal of free radicals. Because the active sites of these enzymes are composed of several amino acids, some amino acids themselves have an ability scavenge free radicals. Therefore, amino acid-type surfactants are promising in terms of supplying AOXs as well as serving as green surfactants with low toxicity and biodegradability[7-10]. Amino acid-type surfactants are diverse, and include those offering anti-aging, antimicrobial, and AOX activities in food, cosmetics, and pharmaceuticals[11-17]. The solubility of AOXs in the emulsion medium is also vital for retarding lipid oxidation in these products. However, there are almost no reports that elucidate the AOX properties of amino acid-type surfactants. We successfully prepared AOX amino acid surfactants from tyrosine, tryptophan, histidine, cystatin, and methionine, and reported their AOX properties considering the amino acid residue[18,19]. It is well known that the physical properties of surfactants are strongly affected by the hydrophobic chain length. Therefore, it is interesting to clarify the effect of the hydrophobic chain length on the AOX properties of surfactants.

In this study, we focused on tyrosine-type surfactants because of their high stability in solid crystals. Tyrosine is among the most insoluble amino acids in water, which is disadvantageous. Therefore, N-acyl amino acid-type surfactants are modified in the form of a water-soluble dipeptide with alanine. Inserting alanine residues enhances the water solubility through a stereochemical advantage[18]. Using several fatty acids with different chain lengths as surfactant materials, four alanyl-tyrosine-type surfactants, C12AlaTyr, C14AlaTyr, C16AlaTyr, and C18AlaTyr, were synthesized. Once prepared, the series of surfactants were analyzed for their AOX activities in the monomer and micellar states.
2 Experimental Procedure

2.1 Materials
Alanine, tyrosine, N-hydroxysuccinimide (HOSu), and dicyclohexylcarbodiimide (DCC) were purchased from Peptide Institute, Inc. (Osaka, Japan). Initially, alkanoyl alanines (CnAla : n = 12, 14, 16, and 18) were obtained using the Schotten-Baumann reaction. All corresponding alkanoyl chlorides (≥98%) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Each CnAla was condensed with HOSu in the presence of DCC, following which the HOSu esters of CnAla were prepared. After recrystallization from ethanol solutions, the esters were reacted with tyrosine in a mixture of tetrahydrofuran and an aqueous solution of sodium carbonate. Alkanoyl alanyl tyrosines were obtained and recrystallized again from the mixture in ethanol-hexane at least twice. Consequently, four alanyl-tyrosine-type surfactants, C12AlaTyr, C14AlaTyr, C16AlaTyr, and C18AlaTyr, were prepared. The purity of the compounds was confirmed to be higher than 98% by HPLC and elemental analysis. In the 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid ammonium salt) (ABTS) assay, we used ABTS and peroxodisulfate purchased from Tokyo Chemical Industry and Wako Pure Chemical Industry, respectively. 2,2-azobis(2-methylpropi- on amidine) dihydrochloride (AAPH) was purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Auramine (N9N9N9N9-tetramethyl-4,4'-diaminodiphenyl ketone hydrochloride, AM) was purchased from Kanto Kagaku Co., Ltd. (Tokyo, Japan) and used as a probe dye to determine the critical micelle concentration (CMC). The concentration of the probe molecule was 1 × 10<sup>-5</sup> M. Every measurement was carried out in phosphate buffer medium at pH 7.4. All other chemicals used for synthesis, purification, and analysis were of analytical grade and used as obtained.

2.2 Physiochemical properties
It is known that the ratio of the fluorescence intensity in an aqueous solution containing no surfactant (I<sub>f</sub>) to that with a surfactant solution (I) increases when AM molecules are solubilized in molecular aggregates such as micelles. The excitation and emission wavelengths of AM were 390 nm and 490 nm, respectively. The fluorescence spectra of AM were recorded using a fluorescence spectrophotometer (Hitachi F 2700, Tokyo, Japan).

2.3 Antioxidative properties
2.3.1 ABTS assay
The AOX capacity was evaluated using the ABTS assay. Equal amounts of 7 mM ABTS aqueous solution and 2.45 mM potassium peroxodisulfate aqueous solution were mixed and placed in the dark for 16 h. Stable ABTS radical cations were obtained. The solution was diluted with 0.1 M phosphate buffer solution (pH 7.4) such that the absorbance at 735 nm was 0.70 ± 0.02 at 25°C and the total amount was 1980 μL. The absorbance was measured 1 minute after dilution, and was considered as the 0 min data. Two minutes after dilution, 20 μL of the surfactant solution was added. The absorbance was measured every minute for 6 min post the addition of genistein. The percentage inhibition was determined using the following equation:

\[
\% \text{ Inhibition} = \frac{A_0 - A}{A} \times 100
\]

Where A<sub>0</sub> is the absorbance at of ABTS radical cation 735 nm after 6 min in the absence of the AOX surfactant, and A is the absorbance at 735 nm after 6 min in the presence of the AOX surfactant. The percentage inhibition was plotted as a function of the genistein concentration. IC<sub>50</sub>, the concentration at which the percentage inhibition was 50%, was determined. The Trolox equivalent antioxidant capacity (TEAC) was calculated using the following equation:

\[
\text{TEAC} = \frac{\text{IC}_{50 \text{ of Trolox}}}{\text{IC}_{50 \text{ of AOX}}}
\]

The IC<sub>50</sub> of Trolox was 10.7 μM in this study.

2.3.2 ORAC Assay
The oxygen radical absorbance capacity (ORAC) assay is extensively used to determine the radical scavenging capacity of AOX materials. This assay has been used to detect the AOX activities of amino acids and their corresponding surfactants. AAPH is a peroxyl radical generator used in this assay to generate radicals in the solution. Fluorescein is utilized to scale the AOX activity by monitoring the fluorescence spectra within the required time range. The oxygen radical absorbance capacity (ORAC) assay is used as a standard AOX material. The area under the curve (AUC) is a measure of the AOX activity, and was calculated by the following equation:

\[
\text{AUC} = f_0 + f_{100}/f_0 + f_{200}/f_0 + f_{300}/f_0 + 
\]

Where \( f_0 \) is the initial fluorescence intensity without any
AOX species and $f_{(0)}$ is the fluorescence reading at any "even time" in between 0 and 1800 s. A higher AUC value indicates higher AOX activity. Finally, the net AUC was calculated as:

$$\text{net AUC} = \text{AUC}_{\text{AOX}} - \text{AUC}_{\text{Blank}}$$  \hspace{1cm} (4)

The AOX properties were articulated by the Trolox equivalent (TE), as used by Han et al. 30

$$\text{TE} = \frac{\text{Slope of the AOX material curve}}{\text{Slope of the Trolox standard curve}} \cdot \frac{\text{net AUC vs conc.}}{\text{conc.}}$$  \hspace{1cm} (5)

To determine the AOX properties of the surfactants in the micellar medium, we used an external surfactant, decanoyl alanine (C12Ala), at 5 mM, which is a higher concentration than the CMC. C12Ala has very little AOX properties, and its freshly prepared micellar solution is used before any measurement. In the micellar system, $f_{(0)}$ is the initial fluorescence intensity without AOX or C12Ala molecules.

3 Results and Discussion

3.1 Critical micelle concentration

The ratio $I/I_0$ of auramine was plotted against the concentration of the surfactant, as shown in Fig. 1, for all systems at 298.2 K. Every $I/I_0$ value increased sharply at a certain concentration. Therefore, the CMC can be determined as the concentration at the break point. The logarithmic values of the obtained CMC are shown as a function of the carbon number of the hydrophobic chain in Fig. 2. A relatively good linear relationship is confirmed, and the slope is almost 0.3, which is the typical value for ionic surfactant systems. However, the CMC of C18-AlaTyr deviates slightly downward from the regression line. This may be caused by the uncertainty in the determination owing to the lowest CMC. Another reason is the possibility of pre-micelle formation. It has been reported that long-chain surfactants tend to form pre-micelles below the CMC 31.

3.2 Antioxidative properties of surfactants

The AOX activity of the AlaTyr-type surfactant can be qualitatively determined as an absorbance decrease in the absorption spectrum at 732 nm, which corresponds to the absorbance of the ABTS* cation. This reduction was manifested as the decolorization of the ABTS* cation from blue/green to a more transparent color as a consequence of the collision of the AlaTyr-type surfactant molecule with the ABTS* cation. The percentage inhibition obtained by the ABTS assay was plotted against the concentrations of Tyr, AlaTyr, and AlaTyr-type surfactants for each system in Fig. 3. Since the percentage inhibition values were greater than 50%, the IC50 values were estimated by interpolation for each experimental result. The IC50 and TEAC values are shown in Table 1. The TEAC of Tyr was approximately 2.3, which is the highest value among all samples, and that of AlaTyr was higher than those of AlaTyr-type surfactants. The TEAC of the C12AlaTyr surfactant had almost the same AOX capacity as that of the dipeptide form of AlaTyr. This suggests that the AOX property of the tyrosine residue is maintained even in the surfactant structure. On
the other hand, the TEAC of the AlaTyr-type surfactant decreased with increasing hydrophobic chain length. Therefore, we concluded that the difference is due to the gaps in the diffusion rate and collision frequency of the tyrosine residue and ABTS radical cation. According to the Stokes-Einstein equation, the diffusion coefficient of a particle is inversely proportional to the viscosity and molecular size. In this study, the surfactant solutions were too dilute to vary the viscosity of the water media. Thus, as the molecular size increased, the diffusion coefficient decreased monotonously. This idea is supported by the fact that the AOX capacity of Tyr is higher than that of AlaTyr. It is observed that a smaller molecular size is favorable for the manifestation of AOX properties. Another possibility is that the longer hydrophobic chain might hinder contact between the tyrosine residue and ABTS radical cation.

The same objectives were investigated using the ORAC assay. To quantify the AOX activity, the area between the blank curve without the AOX and the curve obtained using the individual AOX concentrations is vital. This area is referred to as the net AUC. By plotting the net AUC against the concentration of several materials, we quantified and compared the AOX activities in terms of the TE. The results of the ORAC assay are shown in Fig. 4, according to which, the higher the slope of the straight line in the net AUC vs. concentration plot, the better is the material as an AOX. Tyr was found to have a much higher AOX activity than surfactants. Furthermore, the AOX activity decreased with increasing hydrophobic chain number of the AOX surfactant. These phenomena are consistent with the results of the ABTS assay. TE values from the ORAC assay were

### Table 1

| AOXs       | IC50 / μM | TEAC  |
|------------|-----------|-------|
| Trolox     | 10.7      | 1     |
| Tyr        | 4.55      | 2.34  |
| AlaTyr     | 8.31      | 1.28  |
| C12AlaTyr  | 9.64      | 1.11  |
| C14AlaTyr  | 11.0      | 0.972 |
| C16AlaTyr  | 31.9      | 0.334 |
| C18AlaTyr  | 33.0      | 0.323 |

### Table 2

| AOXs       | TE without micelle | TE with micelle |
|------------|--------------------|-----------------|
| Trolox     | 1                  | –               |
| Tyr        | 0.842              | 0.328           |
| AlaTyr     | 0.502              | –               |
| C12AlaTyr  | 0.415              | 0.478           |
| C14AlaTyr  | 0.271              | 0.321           |
| C16AlaTyr  | 0.128              | 0.336           |
| C18AlaTyr  | 0.015              | 0.378           |
estimated using Eq. (5) and are shown in Table 2 with the results from the ABTS assay. Although the TE from the ORAC assay was lower than that from the ABTS assay, both tendencies of variation were similar. Thus, the difference in the AOX activity is due to the gaps in the diffusion rate and collision frequency of the tyrosine residue and oxygen radical species. While the AOX activities of Tyr, AlaTyr, and C12AlaTyr were higher than that of Trolox in the ABTS assay, all AOX activities of tyrosine and its surfactants cannot exceed that of Trolox in the ORAC assay. It is suggested that the radical scavenging capacity of tyrosine residues is advantageous for radical cations compared to oxygen radicals. In the experimental concentration range, the concentration was higher than the CMC only for the C18AlaTyr system. It may be reasoned that the small gap between the TEAC values of C16AlaTyr and C18AlaTyr is caused by the micellization effect in the C18AlaTyr system.

### 3.3 Antioxidative properties of surfactant in micelles

Micelle formation is a unique feature of surfactants. Since this study considers an AOX surfactant, the AOX properties at the CMC are a crucial consideration. However, the CMCs of these surfactants are too high to perform ORAC measurements. As the external surfactant providing micellar media, we chose C12Ala because it has almost no AOX properties; hence, it does not interfere with the AOX measurements\(^{18,19,26}\). Because the concentration range of these surfactants is sufficiently low compared with the CMC of C12Ala, the surfactants can enter the C12Ala micelles, making it possible to detect any difference in the AOX properties in the micellar region.

The results of the ORAC assay in micellar media are shown in Fig. 5. As mentioned in our previous report\(^{18,19}\), the micellar medium of C12Ala has an apparent AOX activity for the ORAC assay, regardless of whether C12Ala has any AOX properties. This is because the surfactant micelle and fluorescein are negatively charged and AAPH has a positive charge; hence, AAPH is solubilized in the C12Ala micelle, and the collision between oxygen radicals and fluorescein is interrupted by solubilization. Therefore, the intercept of the net AUC plot is not zero even at zero concentrations of AOX materials. Interestingly, the slope of the net AUC vs. concentration curve did not depend on the hydrophobic chain length. The TE values obtained from these curves are listed in Table 2. A comparison of the TE values in micellar media with those in bulk media is shown in Fig. 6. All TE values of the AlaTyr-type surfactant are considerably recovered, while the TE of Tyr drops suddenly in the
micellar media. Notably, the TE growth rate of the AlaTyr-type surfactant increases with increasing carbon number of the hydrophobic chains. This must be the result of the concentration effect in micelles for surfactant-type AOXs. In other words, the micellar media produce a limited reaction site for the peroxide produced from the AAPH cation and AlaTyr-type surfactant. This phenomenon is regarded as a type of micellar catalysis.

Next, we verify the enhancement effect of the micelles more quantitatively. For this, the concentration of each AlaTyr-type surfactant in the mixed micelle was estimated using thermodynamic relations. It is reasonable to assume that ideal mixing of C12Ala and CnAlaTyr occurs in the mixed micelle, because the properties and structures of these surfactants are very close. Therefore, if both CMC values in the pure system are provided, the total monomer concentration of component 2. According to the lever rule, the concentration of CnAlaTyr in the mixed micelle was estimated using the following relations:

\[
\begin{align*}
C'(\text{monomer}) &= C_{\text{MC}1} X_1^n + C_{\text{MC}2} X_2^n \\
\frac{1}{C'(\text{monomer})} &= \frac{X_1^n}{C_{\text{MC}1}} + \frac{X_2^n}{C_{\text{MC}2}} \\
C'(\text{micelle}) &= C'(\text{monomer}) - X_2^n + X_2^m \\
C &= C'(\text{monomer}) + C'(\text{micelle})
\end{align*}
\]

Table 3: Comparison of the mole fraction and concentration of CnAlaTyr surfactants in binary mixed micellar state under the ideal mixing.

|          | CMC / mM | \(X_{\text{AOX}}\) in micelle | \(C_{\text{AOX}}\) in micelle / mM | \(C_{\text{AOX}}\) in Bulk / mM |
|----------|----------|-------------------------------|-----------------------------------|---------------------------------|
| C12Ala   | 3.0      | 0.0018                        | 3.57                              | 1.4                             |
| C12AlaTyr| 0.81     | 0.0018                        | 4.39                              | 0.6                             |
| C14AlaTyr| 0.28     | 0.0022                        | 4.79                              | 0.2                             |
| C16AlaTyr| 0.092    | 0.0024                        | 4.98                              | 0.02                            |
| C18AlaTyr| 0.011    | 0.0025                        |                                    |                                 |

4 Conclusion

The physicochemical and AOX properties of the AlaTyr-type surfactant series were successfully investigated. Excellent water solubility of the above surfactants was achieved by inserting an Ala residue into the amino acid surfactant frame. The ABTS and ORAC assays showed that the AOX activity of tyrosine was maintained in the surfactant molecules. However, the AOX strength of the AlaTyr-type surfactant decreased with an increase in the hydrophobic chain length in the monomer state. In the micellar solution, while the AOX power of amino acids by the ORAC assay remarkably decreased compared to that without the micelle system, the power of the AlaTyr-type surfactant was enhanced. The degree of enhancement of the AOX power was more remarkable with increasing hydrophobic chain length of the AlaTyr-type surfactant. Because the enhancement effect was balanced by the diffusion effect on the surfactant chain length, the difference in the AOX power among the four AlaTyr-type surfactants became vague.

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