Synthesis and Physicochemical Properties of Dipeptide Type Surfactants: I \( N \)-Dodecanoylglutamyl-glutamic acid and glutamyl-aspartic acid

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Abstract: An attempt was made to synthesize dipeptide-type surfactants analogs from glutamic acid and aspartic acid, which are naturally occurring and renewable amino acids. \( N \)-Lauroylglutamic anhydride was used as a raw material for the synthesis. In this study \( N \)-lauroylglutamyl aspartic acid (LGA) and \( N \)-lauroylglutamyl glutamic acid (LGG) were prepared. Physicochemical properties such as the solubility, the lowering surface tensions, the foaming ability, the emulsification potential, and the higher order structure of the surfactant aggregates were discussed with changing the neutralization of the tri-basic carboxylic acid in the surfactants. These surfactants showed an outstanding surface tension lowering ability. Surface tension and cmc of the aqueous solution of LGA increased as the neutralization of three carboxylic acids increased. On the other hand, those of LGG didn’t show the similar behavior. This difference would result from the inequality in the hydrophobicity produced by one more methylene groups between LGA and LGG. It should bring about some changes such as electrostatic repulsion between two negatively charged carboxylate anions, the inter- and intrahydrophobic interactions and the hydrogen bonds. It was confirmed that these surfactants possessed an excellent biodegradability, a high stability for the hydrolysis, high calcium ion stability. Introduction of the peptide linkage that bonds the two amino acids did not affect the hydrolysis stability of the surfactant. Neither LGG nor LGA were hydrolyzed in basic (\( \text{pH} = 10 \)) aqueous solution for one month. At the same time, they showed a high moisturizing effect in addition to a low protein denaturation potential.


Key words: dipeptide type surfactant, amino acid anhydride, glutamic acid, aspartic acid, moisturizing effects, biodegradation, physico-chemical properties

1 Introduction

In recent years, investigation concerning the production of new surfactants from natural resources has been actively carried out. Sugars, amino acids, vegetable oils and corresponding naturally occurring polymers such as carbohydrates, chitin, chitosan and peptides are noteworthy raw materials. The surfactant derived from \( N \)-higher acylated amino acid is a representative example (1-5). These analogues are readily biodegradable, nontoxic, non-allergic and non-irritant against human skin. In other words, they could be called human- and ecologically friendly surfactants. Since Takehara et al. (6-9) have reported the physicochemical properties, such as surface activities, of mono and disodium salts of \( N \)-acylated glutamic acid, plenty of research on the \( N \)-acylated acidic amino acids, such as aspartic and glutamic acid, have been made (10-12).

The acylated amino acid could be regarded as a carboxylic acid containing an amide group in its hydrophobic alkyl moiety. It is well known that solubility of

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the corresponding alkali metal salt of the amide acid is higher than that of the normal acid. The amide group enhances the wetting and foaming ability and increases the solubility, that is, it lowers the Krafft point (10).

In this work, an attempt was made to synthesize dipeptide type surfactant analogues. Two kinds of naturally occurring renewable amino acids, glutamic acid and aspartic acid, were attached to the N-dodecanoylglutamic acid which is industrially available at reasonable price. Introduction of an additional amino acid could be expected to cause an increase of its multifunctionality, such as surface tension lowering ability, resistance to hard water, foaming property, along with the improvement of the biodegradation and the moisturizing ability.

In this paper, we describe the influence of additional amide group and carboxyl groups. Further, relationships between structure and properties of aqueous solution when neutralization degree of three carboxylic groups in the dipeptide surfactants is changed are also discussed.

2 Experimental

2.1 General Measurement

Throughout the preparation of the intermediates and products, Merck precoated TLC plates (Silica gel 60F 254, 0.25 mm, Art 5715) were used for the TLC analysis. For the preparative column chromatography, Wakogel C-200 (Silicagel : 200 mesh) was employed. Melting points were recorded on an MP-J3 melting point apparatus (Yanagimoto Co.) and were uncorrected. IR spectra were taken with a JASCO spectrometer FT/IR-5300 (Japan Spectroscopic Co.). The identification of the structure was confirmed from the NMR spectra and an elemental analysis. 1H- and 13C-NMR spectra were recorded using a JNM-LA 300 (300 MHz for 1H, 75 MHz for 13C) instruments. Chemical shifts are reported in ppm downfield (δ) from an internal standard (TMS in CDCl3, DMSO-d6 and DSS in D2O). Circular dichroism spectra were taken with a JASCO CD spectrometer J-720W1 (Japan Spectroscopic Co.).

2.2 Raw Materials

N-Lauroylglutamic acid monosodium salt was obtained from Ajinomoto Co. Ltd. L-Aspartic acid α, β-dibenzyl ester tosylate (Asp(OBn)-OBn·Tos) and L-glutamic acid α,γ-dibenzyl ester tosylate (Glu(OBn)-OBn·Tos) were supplied by Peptide Institute Inc., and were used without further purification. All other reagents and solvents were of the highest commercial purity available.

Fig. 1 Preparation of the Dipeptide Type Surfactant.
2.3 Synthesis of the Dipeptide Surfactants

The dipeptide type surfactants were prepared from the N-lauroylglutamic anhydride and the corresponding acidic amino acid derivative. Figure 1 shows an outline for the preparation of the dipeptide surfactants. The preparation of N-lauroylglutamyl-glutamic acid 4(n=2), LGG, and its corresponding sodium salt, LGGNa, is described here as an example.

N-Lauroylglutamic anhydride, 2, was synthesized by dehydration with acetic anhydride (13). N-Lauroylglutamic acid 1 (6 g, 18 mmol) was reacted with freshly distilled acetic anhydride (3.7 g, 36 mmol) in 6 mL dry tetrahydrofuran (THF) with stirring at 60°C for 10 h. After THF and excess acetic anhydride were evaporated in vacuo, the crude product was purified by recrystallization from ethyl acetate to yield 2 as a colorless flaky crystal, 4.2 g, yield 75%, mp. 92-94°C. 1H-NMR is summarized in Table 1.

N-Lauroylglutamic anhydride 2 (1.52 g, 4.65 mmol) and Glu(OBn)-OBn·Tos (2.32 g, 4.65 mmol) and triethylamine (0.47 g, 4.65 mmol) were dissolved in 15 mL of THF with stirring. The solution was stirred overnight at room temperature. After removal of solvent, the residue was dispersed in 50% aqueous methanol and washed until the wash solvent became clear. Insoluble material 3(n=2) was collected by filtration. Its 1H-NMR spectrum was consistent with the expected structure.

N-Lauroylglutamyl-glutamic acid, 4(n=2) was obtained by the reductive debenzylation of the dibenzyl ester 3(n=2). The THF solution containing 2.90 g (4.54 mmol) of 3(n=2) was allowed to hydrogenate overnight at room temperature with agitation in the presence of a 10% palladium/carbon catalyst and hydrogen at atmospheric pressure. After filtration of the catalyst, the solvent was removed by evaporation under reduced pressure. The residue was recrystallized from ethyl acetate. Elimination of the benzyl group was confirmed by a 1H-NMR spectrum. The signals corresponding to the benzyl group completely disappeared. The product was greasy and showed a broad melting point at 91-95°C.

Elemental analysis; calculated for 4 (n = 2), C_{21}H_{32}O_{9}N_{2}·1/2H_{2}O: C, 56.52% H, 8.40% N, 5.99%; Found: C, 57.15% H, 8.48% N, 5.76%.

Synthesis of N-lauroylglutamyl-aspartic acid 4(n=1) was similar and could be done as described above. Asp(OBn)-OBn·Tos was used instead of Glu(OBn)-OBn·Tos. The product was hygroscopic and showed broad melting point at 76-80°C.

Elemental analysis; calculated for C_{21}H_{32}O_{10}N_{2}: 1/2H_{2}O: C, 55.61% H, 8.22% N, 6.18%; Found: C, 55.79% H, 8.33% N, 5.99%.

1H-NMR spectra of the dipeptides (4 (n=2 and 1)) are shown in Table 1.

The prescribed amount of aqueous 1M NaOH solution was added to the tri-basic acid type dipeptide 4, in proportion to the required neutralization.

Neutralization degree, N.D., was defined like following equation.

\[
N.D. = \frac{[\text{NaOH}]}{[\text{LGG}]} \text{ or } [\text{LGA}]
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### Table 1 1H-NMR Data of 2 and 4(n=1 and 2).

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2.4 Measurement of Physicochemical Property

Surface Tension Measurement Surface tension was measured by Wilhelmy's vertical plate method with a Model A-3 tensiometer (Kyowa Kagaku Co., Ltd.). The purified dipeptides were dissolved in deionized and distilled water using equipment made of quartz. Conductivity and surface tension of the water used were confirmed to be less than 1 µS/cm and more than 71.0 mN/m, respectively. Adding 1 M NaOH controlled neutralization. The solution was maintained at 25°C. Values were taken until the surface tension was constant for a period of 20-30 min. As lower surfactant concentrations, it usually took longer times to reach equilibrium. Reproducibility of the surface tension measurements is <0.2 mN/m. Results are summarized in Fig. 2[A] for LGG, and Fig. 2[B] for LGA, respectively.

Foaming Test In the studies of foaming test of the dipeptide type surfactant, the semimicro improved TK-method was used as described by Yano and Kimura (16). The summary of the equipment refers to the literature. Foams were prepared by bubbling certain quantity (250 mL) of air through the glass tube of 2 mm inside diameter for a fixed time (60 sec). The volume of foams was measured immediately after the bubbling (V₀) and five minutes later (V₅). The results are summarized in Fig. 4.

Emulsification Emulsification ability was determined by the volume of emulsion layer after shaking of 5 mL of hexane and 5 mL of 25 mM aqueous solution of LGG and LGA under various neutralization degrees. The test was carried out in a test tube with marked lines. The sample solution was set in a thermostat oven at 50°C for fixed time. The values indicate the volume of aqueous solution separated from the emulsion. This experiment was worked up in a manner similar to that of literature (17). The results are summarized in Fig. 5.

Calcium Ion Stability This experiment was done in accordance with the method of Takehara (8) with a little modification. An aqueous solution of 100 mM calcium chloride was added into 10 mL of a 10 mM LGG or LGA aqueous solution until the solution became cloudy with stirring at 40°C. The amount of calcium ion in the resultant solution was indicated as the calculated concentration (ppm) of calcium carbonate. The results are shown in Fig. 6.

Moisturizing Ability The moisturizing ability of surfactant, that is, water-absorbing and water-holding capacity was evaluated as follows: The dried surfactants were maintained in a desiccator whose humidity was adjusted to 80% or 45% at 25°C for prescribed time and the amount of absorbed water was calculated from the increase in weight. The sample in the desiccator was transferred to another desiccator whose humidity was 45% or 0%, respectively, and the weight loss was measured again to evaluate the ability of retaining the water. LGANa and LGGNa were the evaluated samples. Neutralization degree of these surfactants was two. In addition, glycerol and sodium lactate (LacNa), which are representative moisturizing agents, were also tested for the comparison.

Water contents (%) = 100 x (W₀ - W₅) / W₀

where W₀ is weight of the wet sample, W₅ is weight of the dry sample. Results are shown in Fig. 7.

2.5 Biodegradation Test

Biochemical oxygen demand (BOD) was determined with a BOD tester (Model 200 and 100 F; TAITEC Corp., Koshigaya, Japan) by oxygen consumption method basically according to the OECD guidelines for Testing of Chemicals 301C, Modified MITI (Ministry of International Trading and Industry) Test at 25°C (14), using an activated sludge freshly obtained from a municipal sewage treatment plant. The concentration of the surfactant in the incubation media was 25 mg/L. BOD could be calculated according to the equation below.

\[ \text{BOD} = \frac{A - B}{22.4 \times 10^3 \times \left( \frac{298}{273} \right)} \times 32 \times 10^3 \times \frac{C}{C} \]

where A = oxygen consumption volume of sample solution (mL)

B = oxygen consumption volume of blank (mL)

C = weight of tested sample (gram)

Theoretical oxygen demand (ThOD) was defined as the weight of oxygen consumption when one gram of a compound was completely mineralized to water and carbon dioxide.

Then, biodegradation can be calculated as,

\[ \text{Biodegradation} = \frac{\text{BOD}}{\text{ThOD}} \times 100% \]

The results of the biodegradation test are summarized in Fig. 8.

2.6 Hydrolysis Stability

To estimate the stability for hydrolysis, solutions of LGA and LGG were tested.

Each of the dipeptide type surfactants was dissolved in a buffer solution of pH 4 (AcOH/.AcONa) and pH 10 (NaOH/Glycine) and allowed to stand for four
weeks. Concentration of the surfactants was 10 mM. The hydrolysis was ascertained by monitoring the change in HPLC every one week. Analytical conditions were as follows: Column: ODS Inertsil, Eluent: Acetonitrile/Water = 3/1 (v/v), Flow rate: 0.4 mL/min, Wavelength of the UV detector: 204 nm.

The hydrolyzed products were recovered and discussed by 1H-NMR through comparing two spectra of the original surfactant and the hydrolyzed one.

2.7 Protein Denaturation Test

Quantitative measurement of the protein denaturation potency of the dipeptide surfactants was determined using a gel permeation chromatography (GPC). Evaluation was performed in a manner similar to that of Miyazawa (15), that is, the degree of denaturation was obtained quantitatively by calculating how much the peak height of the ovalbumin decreased in a GPC analysis.

Ovalbumin was treated with various kinds of surfactant at different concentration and compared with an untreated ovalbumin. Neutralization degree of LGG and LGA was two. The sample solution was composed of 9 mL of phosphate buffer solution (pH 7) containing 2.25 mg of ovalbumin and 1 mL of surfactant aqueous solution. Ovalbumin was obtained from commercial sources (Sigma Chemicals Inc., Tokyo) and was used, as received, without further purification.

The analytical conditions were as follows: High performance liquid chromatography system composed of Trirotor II with a GPC column (TSK-G3000SW) and with an UV detector (UVDEC-100-III) was used. The detector was operated at 204 nm at the absorption depending on the peptide linkage. Elution solution was prepared from 0.05 M phosphate buffer solution (pH = 7.0) containing 0.15 M sodium sulfate. A sample volume of 10 μL was injected into the HPLC system for analysis. Mobile phase were filtered through 0.45 μm membrane filters prior to use. A mobile phase flow rate of 1 mL/min was used. The results of the protein denaturation test are summarized in Table 2 and Fig. 9.

3 Results and Discussion

3.1 Physicochemical Characteristics of the Surfactants

Surface Activity of the Surfactants In order to confirm the evidence of micellization or aggregation, the relationship between surface tension and concentration was investigated. Surface tension of aqueous solution of the dipeptide surfactants was measured as a function of concentration at 25°C. Figure 2 depicts the surface tension versus log of the bulk phase concentration for an aqueous solution under various degrees of neutralization.

On LGA, critical micelle concentration (cmc) and surface tension at cmc (γcmc) increased with increase of the degree of neutralization. It was confirmed that adsorption of surfactants at air-liquid interface and surface tension lowering ability tend to be contradictory with the degree of neutralization. These results are closely related to their ionization. It is possible that the results shown in Fig. 2 originated from the electron repulsion that grew with the increase of the degree of neutralization. The molecules of the surfactant packed more loosely, when three carboxyl groups in LGA were changed into their corresponding carboxylate anions, and this weakened inter- and intramolecular hydrogen
bonds that cause aggregation of the surfactant molecules.

On the contrary, the correlation mentioned above could not be observed for LGG, that is, cmc and \( \gamma_{\text{cm}} \) of LGG were not influenced by the neutralization degree of the surfactant. This difference was due to the difference between the structure of the second amino acid, glutamic acid and aspartic acid. When the degree of neutralization increases, the intermolecular distance increases. This is due to the increase in electronic repulsion between carboxylate anions. Because intramolecular hydrogen bonds of LGG in an alkaline solution are stronger than those of LGA, the effects of neutralization degree were ambiguous.

To confirm the aggregation state of the surfactants, the CD spectrum was measured. The aqueous solution of 0.2 mM of LGG and LGA were prepared and examined at 25°C under various pH conditions. It is shown in Fig. 3.

In a spectrum of LGG, the spectrum continuously changed from higher molecular ellipticity to lower one in proportion to the degree of neutralization of the sample. It said that the molecular orientation in an aqueous solution was randomized in proportion to the neutralization.

On the other hand, the spectrum of LGA showed the aspect that differed from that of LGG to some extent. In this spectrum the isosbestic point was recognized. The spectrum showed the reverse shapes in neutralized and non-neutralized state. Negative ellipticity was amplified as neutralization decreased in the region over the 215 nm wavelength.

The difference of two spectra depicted that their aggregation structure was obviously different between LGG and LGA. In this study, while it is not possible to conclude what kind of behavior is the difference, the difference obviously comes from the second amino acid. The difference of the number of methylene groups between amino group and carboxyl group brought about the difference in hydrogen bond and hydrophobic interaction, and it made the difference of such spectra.

**Foaming Behavior** Comparing the dipeptide type surfactants and the monopeptide type one, both the foaming ability and stability of the former surfactant were more excellent than that of the latter, as shown in Fig. 4. The foaming ability, \( V_f \), for LGG and LGA was excellent throughout the entire neutralization degree. There was not clear difference for \( V_f \) between LGG and LGA.

Influence of neutralization was clearly observed for the foaming stability. \( V_f \) of LGA decreased as neutralization increased. On the other hand, that of LGG was stable under any neutralization degree.

**Emulsion Stability** Figure 5 shows the volume of aqueous solution separated from the emulsion phase. The emulsion stability decreased with an increase in the neutralization degree. This tendency was more remarkable for LGG than for LGA. The aqueous solution of the dipeptide type surfactants had almost equal emulsifying powers to those of LGNa. LGG gave the best emulsion stability when its three carboxyl groups were free.

**Calcium Ion Stability** The 10 mM aqueous solution of the dipeptide type surfactants showed high stability against calcium ion, in spite of the carboxyl groups of the surfactant were free or sodium salt. Every dipeptide type surfactant tested was stable below the concentration of 1000 ppm calculated as calcium carbonate, as shown in Fig. 6. Analyzing the results in detail, it was confirmed that form of carboxyl group is significant to exhibit the stable resistance to hard-water. Especially, the dipeptide type surfactant that possessed three free carboxyl groups in a molecule showed excellent effects about three orders of magnitude over that of the ordinary soap. Its stability decreased as the neutralization
degree of three carboxyl group increased. Number of carboxyl groups in a surfactant is another fundamental important factor to determine the calcium ion stability. LGG and LGA that possess three carboxyl groups showed more pronounced stability further than LGNa that possesses only one carboxyl group.

Moisturizing Ability As Fig. 7 demonstrates, LGANa showed the most excellent moisturizing ability among the peptide type surfactants in this study. Its water absorbing and holding capacity was comparable to that of sodium lactate (LacNa) and glycerol, which are representative natural moisturizing ingredients. The moisturizing ability of the surfactants neutralized with

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Fig. 4 Foaming Ability (V₀) and Foam Stability (V₅) of LGG and LGA under Various Neutralization Degree.

Fig. 5 Emulsion Stability of Surfactants under Various Neutralization Degree [LGG], [LGA] = 25 mM, 50°C.

Fig. 6 Calcium Ion Stability of the Surfactants. * indicates the value in the reference(8)
sodium hydroxide was significantly higher than the corresponding surfactant possessing free carboxylic group. Synthetic LGANa and LGGNa demonstrated excellent moisturizing ability than LGNa that was used as the raw material. It seems like that the number of carboxyl group in a surfactant molecule is one of the indispensable factors for showing the moisturizing ability. Dipeptide type surfactant has three carboxyl groups and lauroylglutamic acid (LG) that was used as the raw material has two. The difference of the number of the carboxyl groups is the main reason for the difference of this moisturizing property. The amide linkage introduced for the second amino acid caused no obstruction for maintaining the moisturizing ability.

3.2 Biodegradation and Hydrolysis of the Dipeptide Type Surfactants

BOD measurement is a useful and advisable method to predict the potential aerobic biodegradability of the organic compound. Figure 8 shows the biodegradation of the dipeptide type surfactants, LGG and LGA. The decomposition of both surfactants started immediately after contact with microorganisms. In six days the biodegradation of both surfactants reached 60% that is a standard for easy decomposability. This biodegradability was equivalent to that of the lauroylglutamate. It is clear that the newly introduced amide linkage would not impede the biodegradation, and the dipeptide type surfactants will be rapidly decomposed in the environment. The structural difference between glutamic acid and aspartic acid as the second amino acid showed no influence on the biodegradation.

The experimental results relating to the hydrolysis indicated that these peptide type surfactants were very stable both in acidic and basic aqueous surroundings. They could be entirely recovered from the aqueous solution in the same state as before the hydrolysis. Proton NMR spectra and HPLC data explained that there were no distinction between the original surfactants and the resulting compounds.

3.3 Protein Denaturation Test

The GPC analysis proved that every peptide type surfactant studied in this work, such as lauroylglutamic acid and its sodium salt (LG, LGNa) and dipeptide type surfactants (LGG, LGA) and corresponding sodium salts with neutralization degree of two (LGGNa, LGANa) were not stimulant against ovalbumin compared with SDS and TMAC that were also studied along with the peptide surfactants. The peaks of untreated ovalbumin were observed for about two weeks and their chromatograms were found to be nearly constant in height and area. Table 2 shows the influence of types of surfactants and the coexisting period on the denaturation of ovalbumin when the protein coexisted with the surfactants at its concentration of 10 mM. When ovalbumin came into contact with the surfactant, the protein immediately denatured, although the magnitude of denaturation was dependent on the kind of the surfactant. It was confirmed that all of the peptide type surfactant showed low denaturing potency whether its carboxyl group was free or it was sodium carboxylate. On the other hand, the influence of sodium dodecyl
Table 2  Influence of Time and Surfactants on the Ovalbumine Denaturation.

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<tr>
<td>LGANa</td>
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<tr>
<td>LGNa</td>
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<td>Lauric acid Na</td>
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<tr>
<td>SDS</td>
<td>65</td>
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<tr>
<td>TMAC</td>
<td>68</td>
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Conc. of Surfactant : 10 mM

Fig. 9  Influence of Surfactant Concentration on the Denaturation of Ovalbumin.

sulfate (SDS) and N,N,N-trimethyl-N-tetradecylammonium chloride (TMAC) on denaturing ovalbumin was outstanding. Among the amino acid derivative surfactants, the dipeptide type surfactants showed lower denaturation potency than the monopeptide type, LGNa. The results indicate that the newly created amide linkage did not give an undesirable denaturing property to the surfactant. The influence of storage time was insignificant when the concentration of surfactant was 10 mM.

Figure 9 indicates the relationship between the denaturation of ovalbumin and the concentration of the surfactants. It was clearly confirmed that the denaturation potency of the surfactant was directly proportional to concentration. When the effect of the concentration was observed, the effect of the concentration of SDS and TMAC was more remarkable than that of LGGNa and LGANa. In the experiment of the SDS and TMAC, 1 mM of the surfactant concentration gave only 10—20% of denaturation while 10 mM concentration gave more than 80% denaturation. On the other hand, the effect of rise of concentration of the surfactant derived from amino acid, TGGNa and TGANa on protein denaturation was relatively small.

Acknowledgement

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References


