Lysozyme−Glucose Stearic Acid Monoester Conjugate Formed through the Maillard Reaction as an Antibacterial Emulsifier

Koji Takahashi,[†] Xue-Fang Lou,[†] Yasuhiro Ishii,[‡] and Makoto Hattori*,[†]

Department of Applied Biological Science and Scleroprotein and Leather Research Institute, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

Lysozyme−glucose stearic acid monoester (HEL−GE) conjugate was prepared through the Maillard reaction as an antibacterial emulsifier. The molar ratio of GE to HEL was 1:1. The isoelectric point was 6−7, which is lower than that of native HEL. Spectroscopic studies indicated that the α-helix content was slightly lower but the conformation around Trp had not changed and that the surface of the conjugate was covered with the GE moiety. The conjugate maintained ∼53−57% of the enzymatic activity of native HEL at 40−60 °C and exhibited considerable resistance to proteolysis. The denaturation temperature of the conjugate was ∼74 °C, somewhat higher than that of control HEL, whereas the enthalpy was about one-third of that of control HEL. The emulsifying activity of the conjugate and the emulsion stability were much enhanced compared to those of native HEL, and the conjugate maintained ∼70% of the bactericidal activity.

Keywords: *Lysozyme; protein conjugation; fatty acylated saccharide; Maillard reaction; emulsifying ability; antimicrobial activity*

INTRODUCTION

Hen egg lysozyme (HEL) is well-known as an enzyme that has the ability to cause lysis of bacterial cells (Imoto et al., 1972). The protein has a molecular weight of 14400 (129 amino acid residues), and it has a high isoelectric point (p*I* 11.0) due to a high content of His, Arg, Lys, Gln, and Asn (Canfield, 1963). The three-dimensional structure of crystalline HEL has been determined by X-ray crystallography (Blake et al., 1965), and the structure in solution has been studied in detail by NMR spectroscopy (Smith et al., 1991, 1993). It is comprised of two domains: one domain has four α-helix regions (A, residues 4−15; B, residues 24−36; C, residues 88−99; D, residues 108−115), and the other has an antiparallel *β*-sheet region (residues 41−60) and a loop region (residues 61−78). X-ray crystallography and NMR findings indicate that the folded structure of the protein is similar in crystals and in solution, whereas the mobility of the side chains in solution, especially those on the surface of the molecule, is greater than that in the crystal structure (Smith et al., 1993). Because HEL has four disulfide bridges (^{6}Cys−^{127}Cys, ^{30}Cys−^{115}Cys, ^{64}Cys−^{80}Cys, and ^{76}Cys−^{94}Cys), the flexibility of the main chain is considered to be very restricted even in solution, resulting in considerably high structural stability, and this is primarily related to the interfacial properties of the protein such as the emulsifying ability and foaming ability.

Because HEL exhibits little emulsifying ability, some efforts have been made to improve it. Conjugation of HEL with a neutral polysaccharide such as dextran, galactomannan, or xyloglucan by the Maillard reaction and polymannosylation by genetic modification were found to be effective in improving the emulsifying activity of HEL and the emulsion stability, probably due to the high hydration capacity of the attached polysaccharide chains (Nakamura et al., 1991, 1992, 1993; Shu et al., 1996). In addition, the conjugates exhibited broad-spectrum lytic action against not only Gram-positive bacteria but also Gram-negative bacteria when the cells were heated at 50 °C for 20 min with them. Ibrahim et al. (1991, 1993) reported that fatty acylated HEL prepared by using *N*-hydroxysuccinimide showed lytic action against both Gram-positive and Gram-negative bacteria without the need for heating, and its antibacterial effectiveness was dependent on the size of the hydrophobic moiety. The emulsifying activity was improved, whereas the emulsion stability was not. All attached moieties in the conjugates mentioned above were noncharged materials. Conjugation of HEL with a polyanion such as carboxymethyl dextran resulted in the disappearance of the lytic action despite maintenance of the enzymatic activity and improvement of the resistance to proteolytic digestion (Hattori, 1994); therefore, the marked decrease in the positive charge of HEL that occurs upon its conjugation with a polyanion is considered to be unfavorable. These results thus suggest that combined treatment consisting of glycosylation with a neutral saccharide and fatty acylation may be effective to achieve improvement of the emulsifying activity of HEL and the emulsion stability as well as maintenance of the lytic activity.

Glycolipid, having soluble saccharide and fatty moieties, is considered to be one of the kinds of substances which when conjugated with HEL could potentially serve to achieve the same sort of improvement as that expected to be attained by the combination of conjugation with neutral saccharide and fatty acylation. However, natural glycolipid does not have a saccharide moiety with a reducing terminus, which would allow

* Author to whom correspondence should be addressed (telephone +81-42-367-5879; fax +81-42-360-8830; e-mail makoto@cc.tuat.ac.jp).

[†] Department of Applied Biological Science.

[‡] Scleroprotein and Leather Research Institute.

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conjugation with a Lys residue in HEL through a glycosylamine linkage in the Maillard reaction. Because some fatty acylated saccharides have a reducing terminus unlike glycolipids, such substances may be suitable as modifiers for this purpose. In the present study, glucose stearic acid monoester (6-*O*-stearyl-D-glucose, GE) as a fatty acylated saccharide was thus conjugated with HEL by the Maillard reaction, and the structural features and unique properties of the HEL−GE conjugate as an antibacterial emulsifier were examined.

MATERIALS AND METHODS

Materials. Lysozyme (HEL) was purchased from Wako Pure Chemicals Industry Ltd. (Osaka, Japan). 6-*O*-Stearyl-D-glucose (glucose stearic acid monoester, GE) prepared by esterifying glucose with stearic acid chloride in pyridine/dioxane and recrystallizing the esterified product from methyl ethyl ketone/methanol was obtained from Mitsubishi Chemical Corp. (Tokyo, Japan).

Preparation of the HEL—GE Conjugate. GE (0.6 g) was well dispersed in 100 mL of water with stirring at 40−50 °C; 1.2 g of HEL was dissolved in this suspension at room temperature, and then it was lyophilized. The dried mixture was incubated at 60 °C at a relative humidity of 79% for 7 days. The reaction mixture was washed with 400 mL of ethanol to eliminate free GE and then dissolved in water. After filtration with a membrane filter (0.45 μm), the HEL−GE conjugate was salted-out with ammonium sulfate at a final concentration of 1 M, and the precipitate was recovered by centrifugation at 8000 rpm for 10 min at 4 °C. The precipitate was then redissolved in 5 M urea. After dialysis against distilled water and lyophilization, the HEL−GE conjugate was obtained. The control sample was obtained by incubating HEL without GE and carrying out the same treatment.

Isoelectric Focusing. Isoelectric focusing of the HEL−GE conjugate was performed using the Pharmacia Phast System TM (Kramlova et al., 1986), and the protein bands were detected by staining with Coomassie Brilliant Blue.

Quantitative Measurement of Protein. The amount of HEL in the conjugate was determined according to the procedure described in a previous paper (Yang et al., 1998) based on the method of Jaenicke (1974). In brief, a sample containing 0.1−10 μg of protein was mixed with 34 μL of 37% $HC1O_4$ in a test tube and heated to evaporate water at 205−215 °C. The test tube was covered by a glass marble and heated for 20 min. After the contents had cooled to room temperature, 0.5 mL of distilled water was added and mixed well with 0.5 mL of phenol reagent (2% phenol containing 0.01% sodium nitroprusside); 0.2 mL of hydrochlorite reagent (0.02 M NaOCI in 2.5 M NaOH) was then added, and the mixture was kept at room temperature for 20 min prior to the measurement of the absorbance at 578 nm. An ammonium sulfate solution (10^{-2} M) was used as a nitrogen standard.

Quantitative Measurement of Lipid. Fatty acid methyl esters were prepared from the HEL−GE conjugate (5 mg) by methanolysis in 4 mL of 5% hydrogen chloride methanol solution in a screw-capped test tube incubated at 100 °C for 4 h. After the contents had cooled to room temperature, 0.5 mL of distilled water was added, the methyl esters were then extracted three times with hexane:ether (1:1, v/v), and the solvents were removed by evaporation. The sample was dissolved in hexane and then analyzed by gas chromatography using a Shimadzu GC 4CM apparatus (Kyoto, Japan) with a DEGS Chromosorb WAW column (GL Science, Tokyo, Japan). After on-column injection (240 °C), the oven temperature was held at 190 °C. Helium was used as the carrier gas at a flow rate of 450 mL/min, and the eluting compounds were detected by FID. *n*-Heptadecanoic acid was used as an internal standard.

Quantitative Measurement of Free Amino Group. The free amino group content of the HEL−GE conjugate was determined according to the method of Haynes et al. (1967) using 2,4,6-trinitrobenzenesulfonate (TNBS). In brief, 250 μL of the conjugate solution (0.5 mg/mL as the protein concentration), 250 μL of 0.1% TNBS, and 250 μL of 5 M $NaHCO_3$ were mixed and incubated at 40 °C for 2 h. After the addition of 250 μL of 10% SDS and 125 μL of 1 M HCl, the absorbance was measured at 335 nm. The molar extinction coefficient was taken to be 1.4×10^4 mol^{-1} cm^{-1}.

SDS—PAGE. SDS−PAGE was performed according to the method of Laemmli (1970) using 15% acrylamide separation gel, and the gel was stained with Coomassie Brilliant Blue.

CD Spectra. CD spectra of the HEL−GE conjugate (1.0 mg/mL as the protein concentration) in distilled water was recorded at 25 °C with a JASCO J-720WI (Tokyo, Japan) automatic recording spectropolarimeter in a cell of 1.0 mm path length.

Fluorescence Measurement. Fluorescence was measured by means of a Shimadzu RF-5300PC (Kyoto, Japan) fluorescence spectrophotometer: The intrinsic fluorescence of the HEL−GE conjugate dissolved in distilled water at 1 μg/mL (as the protein concentration) was measured at an excitation wavelength of 283 nm.

Measurement of Enzyme Activity. The enzyme activity of the HEL−GE conjugate was measured according to the procedure described in a previous paper (Hattori et al., 1994) based on the method of Nanjo et al. (1988). In brief, the reaction mixture (1.5 mL) containing 0.28 mM *p*-nitrophenyl penta-*N*-acetyl-β-chitopentaoside, 0.2 unit of β-*N*-acetylhexosaminidase, and 50 μg of the conjugate as a protein in 0.1 M citrate buffer (pH 5.0) was incubated at 37 °C for 15 min. After the enzymatic reaction had been stopped by the addition of 1.5 mL of 1.0 M Na_2CO_3, the absorbance was measured at 405 nm. One unit of enzyme activity was defined as the amount liberating 1 μmol of *p*-nitrophenol in 1 min.

Pronase Digestion of the HEL—GE Conjugate. The HEL−GE conjugate (1 mg) was dissolved in 1 mL of 50 mM Tris-HCl buffer (pH 7.5), and Pronase (EC 3.4.24.4, 10000 units; Wako Pure Chemicals Industry) dissolved in the same buffer (1 mg/mL) was added. The reaction mixture was incubated at 37 °C, and after 6 h, 0.1 mL was removed for measurement of the enzyme activity as described above.

Differential Scanning Calorimetry (DSC). The HEL−GE conjugate was dissolved in distilled water at a protein concentration of 10 mg/mL, and 50 μL of this solution was sealed in a silver DSC capsule. DSC was performed to determine the denaturation temperature and enthalpy using a Seiko SSC-5020 DSC 6100 apparatus (Tokyo, Japan) as previously described (Takahashi et al., 1988; Hattori et al., 1994) except for a heating rate of 5 K/min. DSC was calibrated with biphenyl, benzoic acid, and indium as the thermal standards. Water was used as a reference, and an Ag capsule pretreated at 250 °C for 10 min was used to prevent the exotherm of the untreated Ag capsule at ∼170 °C.

Evaluation of the Emulsifying Activity. The emulsifying activity was evaluated according to the method of Pearce and Kinsella (1978). The HEL−GE conjugate was dissolved in 0.1 M acetate buffer (pH 4.5 or 6.2) at a protein concentration of 1 mg/mL. One milliliter of corn oil was emulsified with 3 mL of this conjugate solution at 25 °C by means of a Polytron PTA-21 (Kinematica, Switzerland) for 1 min at 24000 rpm. A 100 μL portion of the emulsion was taken from the bottom of the test tube and diluted to 5 mL with 0.1% SDS solution, and then the absorbance was measured at 500 nm. The emulsifying activity was determined as the emulsifying activity index (EAI), which was calculated by means of the following formula: $EAI = 2T/\phi c$, where $T = 2.3A/I$ [A being the absorbance at 500 nm immediately after emulsification and I (light pass) $= 10^{-2}$ m] and c is the concentration of protein, with ϕ (oil phase volume) $= 0.25$].

Microorganisms. *Micrococcus luteus* IFO 3333, *Staphylococcus aureus* IFO 14462, *Bacillus cereus* IFO 13690, *Escherichia coli* IFO 3301, and *Klebsiella pneumoniae* IFO 14438 were obtained from the Institute for Fermentation (Osaka, Japan) as test organisms. These strains suspended in 302 medium (10 g of polypeptone, 2 g of yeast extract, 1 g of $MgSO_4$, 20 g of agar, and water to 1 L) without agar were spread on slants of Sabouraud medium (25 g of polypeptone, 12.5 g of yeast

extract, 7.5 g of glucose, 5 g of NaCl, 20 g of agar, and water to 1 L) and cultured at 30 °C. After the cells had been subcultured twice on slant cultures, the strains were preserved at 5 °C before use in antibacterial assays.

Antibacterial Assays. Cells precultured in Sabouraud medium without agar for 18 h at 35 °C were harvested from the culture medium by centrifugation at 4 °C at 10000 rpm for 10 min. After two washings of the cells with 0.7% NaCl by centrifugation under the same conditions, the cells were resuspended in 0.7% NaCl at a cell density of 10^5 cells/mL as determined by referring to a curve of the absorbance at 600 nm versus cell number, drawn previously. The HEL−GE conjugate was added to the cell suspension (9 mL) at a final concentration of 0.05 mg/mL as a protein and incubated at 50 °C with shaking at 90 times per minute. After a given incubation time, 1 mL of the suspension was taken in a sterile tube and cooled to room temperature in an ice bath. Serial 10-fold dilutions in 0.7% NaCl were prepared, and 0.1 mL portions were spread onto agar plates (Sabouraud medium). Colonies were counted after incubation at 35 °C for 24 h.

RESULTS AND DISCUSSION

Chemical Features of the HEL—GE Conjugate. Incubation of HEL and GE at 60 °C at 79% relative humidity for 7 days resulted in pale browning, indicative of the progress of the Maillard reaction. Isoelectric focusing showed that the HEL−GE conjugate obtained had a relatively broad isoelectric point (p*I* 6−7), lower than that (p*I* 11**)** of native HEL. These results strongly suggested that HEL and GE were covalently bound through the Maillard reaction. The free amino group content of the HEL−GE conjugate was determined to be 6.0 mol/mol of HEL, as compared with the reactive free amino group content of native HEL, which is considered to be 7 mol/mol of HEL. The results of gas chromatography indicated that only stearic acid was detectable in the conjugate and that the conjugate had 1.6 mol of stearic acid/mol of HEL, suggesting that there was a small amount of free GE remaining. The relative molecular weight of the conjugate was estimated to be 14800 by SDS−PAGE. A small amount of a component corresponding to the dimer was detected, and this was observed also in the case of the control HEL preparation. The molecular weight of GE is 446. From these results, the molar ratio of HEL to GE in the HEL−GE conjugate was determined to be 1:1.

Structural Features of the HEL—GE Conjugate. The far-UV CD spectra of control HEL and native HEL showed almost the same patterns as shown in Figure 1, indicating that 5 M urea treatment resulted in no substantial change in the secondary structure of HEL. The α-helix content of native HEL was 24.6%, which was lower than the value (34.9%) calculated on the basis of the results for HEL in solution obtained by NMR (Smith et al., 1993). The HEL−GE conjugate showed a different CD spectrum and a somewhat low α-helix content (21.3%).

The intrinsic fluorescence of these samples was measured in water at an excitation wavelength of 283 nm. The fluorescence emission spectra of these samples showed the same maximum fluorescence wavelength (340.2 nm), as shown in Figure 2. Because the excitation wavelength (275 nm) and the maximum emission wavelength (303 nm) for Tyr are very different from those (279 and 348 nm, respectively) for Trp (Teale and Weber, 1957), in this experiment, the intrinsic fluorescence is considered to be principally detected as that of the Trp residue. Consequently, the conformation around the Trp residue in the case of these samples was

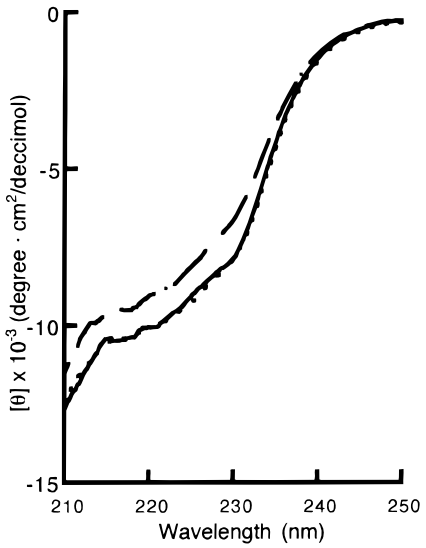

Figure 1. CD spectra of the HEL−GE conjugate (1.0 mg/mL as the protein concentration) in distilled water measured at 25 °C in a cell of 1.0 mm path length: (—) native HEL; (- - -) urea-treated HEL; (— —) conjugate.

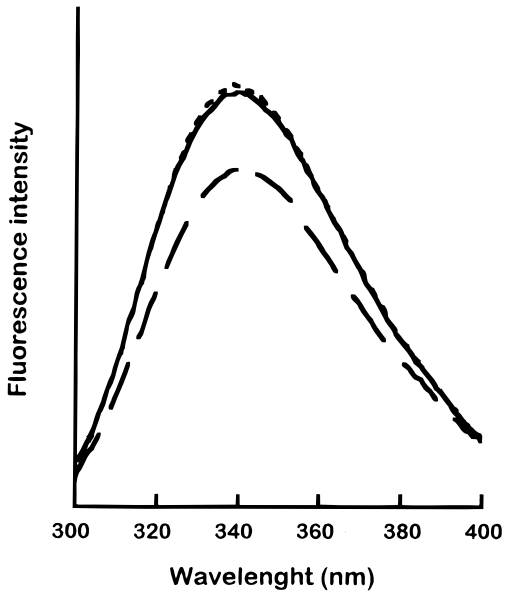

Figure 2. Intrinsic fluorescence of the HEL−GE conjugate dissolved in distilled water at 0.001 mg/mL (as protein) measured at an excitation wavelength of 283 nm: (—) native HEL; (- - -) urea-treated HEL; (— —) conjugate.

considered to be almost the same. In particular, control HEL and native HEL exhibited essentially the same spectrum corresponding to the CD spectra. However, the fluorescence intensity of the conjugate was significantly lower than that of control HEL or native HEL. This lower fluorescence intensity indicates that the residue in GE that is bound to Lys of HEL shielded a certain Trp residue. Similarly, the saccharide chain of the HEL−carboxymethyl dextran conjugate prepared using water soluble carbodiimide was found to exhibit a good shielding effect (Hattori et al., 1994). Shu et al. (1996) reported that the most sensitive site in HEL in terms of participating in the Maillard reaction in a dry state such as at 79% relative humidity was ^{97}Lys in the α-helix region ^{88}Ile−^{99}Val. The conjugation with GE resulted in some decrease in the α-helix content of HEL as described above. It is thus thought that GE is probably attached to ^{97}Lys and shielded near the ^{108}Trp

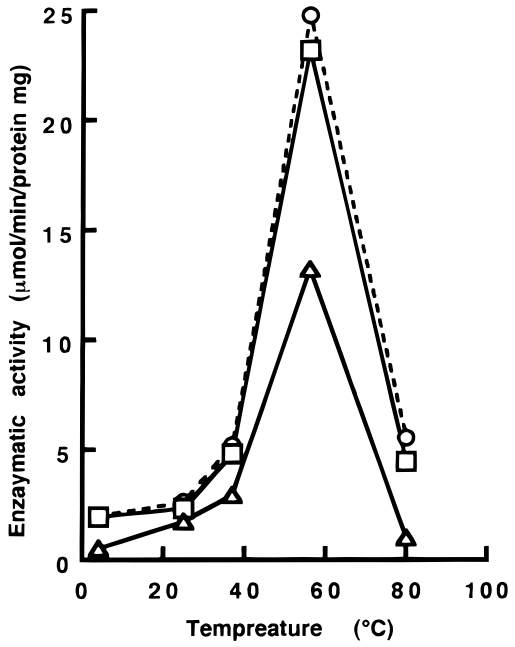

Figure 3. Effect of temperature on the enzymatic activity of the HEL-GE conjugate: (○) native HEL; (□) urea-treated HEL; (△) conjugate. The reaction mixture (1.5 mL) containing 0.28 mM *p*-nitrophenyl penta-*N*-acetyl-*β*-chitopentaoside, 0.2 unit of *O,N*-acetylhexosaminidase, and 50 *μ*g of the HEL−GE conjugate as a protein in 0.1 M citrate buffer (pH 5.0) was incubated at the indicated temperature for 15 min. After the enzymatic reaction had been stopped with 1.5 mL of 1.0 M Na_2CO_3, the absorbance was measured at 405 nm.

residue in three dimensions, as shown in a stereogram of HEL in solution reported by Smith et al. (1993).

Effects on Enzymatic Activity. The effect of reaction temperature on the enzymatic activity of the HEL−GE conjugate is shown in Figure 3. Control HEL exhibited the same enzymatic activity as native HEL at all temperatures and showed the highest activity at 60 °C, because the optimum temperature for native HEL was ∼50 °C. This suggests that no substantial structural change occurred as a result of 5 M urea treatment. However, the enzyme activity of the conjugate was markedly decreased; at 40−60 °C ∼53−57% and at 4 or 80 °C ∼19−27% of the activity of native HEL was maintained. Because GE cannot be conjugated with the active site of HEL (^{35}Glu and ^{52}Asp) through the Maillard reaction, the decrease in enzymatic activity was considered to be due to steric hindrance in the HEL−GE conjugate.

HEL and the HEL−GE conjugate were digested with Pronase at 37 °C for 6 h, and thereafter the enzymatic activity was measured. The residual activities of control HEL and native HEL were 23.2 and 25.6% of the control level without Pronase digestion, respectively, indicating substantial susceptibility to protease digestion. In contrast, the residual activity of the conjugate was 64.6%, showing notable resistance to Pronase digestion. Presumably, GE conjugated with HEL considerably inhibited contact with the protease. Conjugation with GE may be useful in the development of a combined application of HEL and protease.

Thermal Behavior of the HEL—GE Conjugate. The thermal behavior of native HEL, control HEL, and the HEL−GE conjugate was evaluated by DSC. Native HEL denatured at ∼76 °C (T_p) (Table 1), consistent with the previous results (Hattori et al., 1994), whereas

Table 1. Denaturation Temperature and Enthalpy of the HEL−GE Conjugate Evaluated by DSC

	denaturation temp (°C)			enthalpy (mJ/mg)
	T_o	T_p	T_c	
native HEL	69.4	76.4	82.9	37.7
control HEL	65.8	72.4	77.8	39.9
HEL−GE conjugate	66.2	73.6	81.1	13.2

[a] Mean value of duplicate experiments.

control HEL became denatured at ∼72 °C. Because the dissolution in 5 M urea resulted in no changes in conformation as determined from the CD spectra, the intrinsic fluorescence, and the enzymatic activity as described above, it seems that the decrease in the denaturation temperature of control HEL may be due to some distortion of the tertiary structure, except for the conformation around the Trp residue or the active site. The DSC curve for the HEL−GE conjugate was considered to reflect the thermal denaturation behavior of HEL in the conjugate, because GE melted in a higher temperature range (∼89−133 °C) than the denaturation temperature of native HEL. T_p of the HEL−GE conjugate was ∼74 °C, which was a little higher than that of control HEL due to the increase in hydrophobicity resulting from its conjugation with GE. However, the enthalpy of the HEL−GE conjugate markedly decreased as compared with that of native or control HEL. One of the plausible reasons is the change in the α-helix in HEL estimated from CD measurement. The other reason is considered to be the concomitance of the exothermal change due to the molecular ineteraction and aggregation through the stearic acid residue. Further investigations involving DSC analysis using a dilute solution are necessary.

Emulsifying Ability of the HEL—GE Conjugate. The emulsifying ability of the HEL−GE conjugate was evaluated on the basis of the absorbance of emulsions of corn oil and the conjugate solution at pH 4.5 and 6.2. As shown in Figure 4, the emulsions with control HEL showed almost the same emulsifying ability as native HEL, indicating no influence of urea treatment in sample preparation. The EAI of the conjugate was 125 m^2/g at pH 4.5, which was 2.2 times greater than that of control HEL (58 m^2/g). Even at pH 6.2 corresponding to the p*I* of the HEL−GE conjugate, the HEL−GE conjugate showed a high EAI (98 m^2/g) as compared with control HEL (59 m^2/g). The emulsion stability was expressed as the absorbance of the emulsions after 30 min, when the emulsions had nearly attained equilibrium. The emulsion stability of the HEL−GE conjugate was 1.7 times and 6.9 times greater than those of control HEL at pH 4.5 and 6.2, respectively. It was thus concluded that the conjugation of GE with HEL at a 1:1 molar ratio is effective in markedly improving both the emulsifying activity of HEL and the emulsion stability. Ibrahim et al. (1991) reported that HEL acylated with the *N*-hydroxysuccinimide ester of palmitic acid at a ratio of above two palmitoyl residues (C_{16}) per HEL molecule resulted in improvement of the emulsifying activity at the same HEL concentration (0.1%) as that in our experiments, whereas no improvement in emulsion stability was observed. In the present study, because HEL in the conjugate has a relatively long extended aliphatic chain (C_{18}) bound through a glucosylamine linkage between a Lys residue and GE, HEL is considered to be anchored with the stearic acid residue in a state where it is forced up from the oil

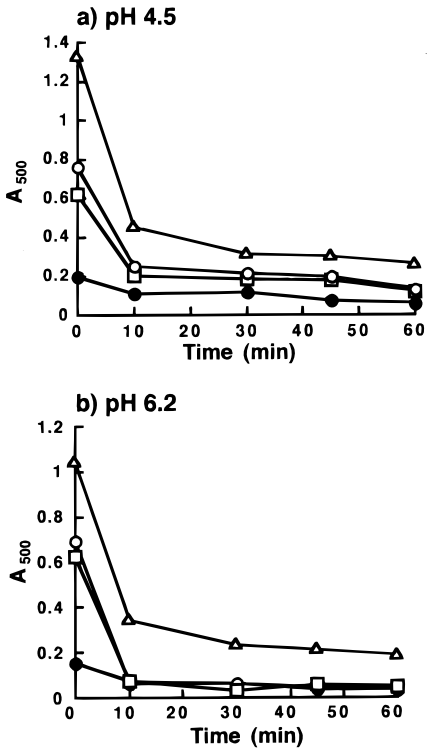

Figure 4. Emulsifying ability of oil-in-water emulsions prepared with the HEL−GE conjugate: (●) control (without protein); (○) native HEL; (□) urea-treated HEL; (△) conjugate. The oli-in-water emulsion prepared [corn oil/0.1% conjugate (as protein) solution, 25:75 (v/v)] was held at 25 °C. The emulsion was 50-fold diluted with a 0.1% SDS solution, and the absorbance at 500 nm was measured.

droplet surface by the glucose residue. Consequently, it is considered to be possible that the HEL−GE conjugate could efficiently become oriented to the surface of oil droplets due to the given mobility of the protein moiety as compared with the conjugate having only palmitoyl residues without a certain glucosidic spacer, resulting in more stable encapsulation of the oil droplet surface and electrostatic repulsion among oil droplets.

Antibacterial Action. The antibacterial action of the HEL−GE conjugate was examined by viable count using three strains of Gram-positive bacteria (*M. luteus* IFO 3333, *S. aureus* IFO 14462, and *B. cereus* IFO 13690) and two strains of Gram-negative bacteria (*E. coil* IFO 3301 and *K. pneumoniae* IFO 14438). Preliminary examination of the antibacterial action of HEL in the concentration range of 0.001−10 mg/mL by the paper disk method showed sufficient inhibition *M. luteus* at 0.01−1 mg/mL, and the optimum concentration of HEL was determined to be 0.05 mg/mL, which was $^1/_{10}$ of the concentration used in the antibacterial assay of HEL acylated with the *N*-hydroxysuccinimide ester of palmitic acid (Ibrahim, 1991). As shown in Figure 5, the conjugate exhibited antibacterial activity against *M. luteus*, and the potency was ∼70% of that of native HEL. However, the HEL−GE conjugate did not exhibit antibacterial activity against the other four strains tested. When the HEL−GE conjugate concentration was increased to 0.075 mg/mL as a protein, the HEL−GE conjugate completely killed *M. luteus* at 10^5 cells/mL within 20 min. Nakamura et al. (1991, 1992) reported that HEL conjugated with dextran or galactomannan through the Maillard reaction showed good antibacterial

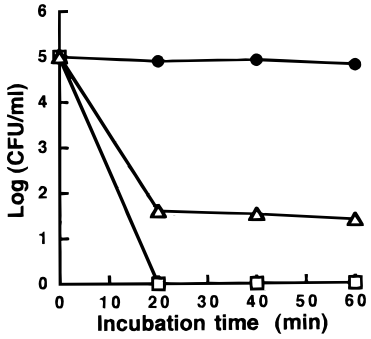

Figure 5. Antimicrobial action of the HEL−GE conjugate: (●) control (without protein); (□) urea-treated HEL; (△) conjugate. *M. luteus* cells precultured in Sabouraud medium and washed with 0.7% NaCl were suspended in 0.7% NaCI at a cell density of 10^5 cells/mL, the HEL−GE conjugate was added to the cell suspension (9 mL) at a final concentration of 0.05 mg/mL (as protein), and the mixture was incubated at 50 °C for the indicated time. One milliliter of the suspension was removed for enumeration of vial cells, and colonies were counted after incubation at 35 °C for 24 h.

activity against Gram-negative bacteria such as *E. coil* as well as Gram-positive bacteria such as *B. cereus*, and the broad spectrum of antimicrobial activity was considered to be probably due to the excellent surface activity of the conjugate. The fatty acylated HEL also showed antibacterial activity against *E. coli* (Ibrahim et al., 1991, 1993), and this was considered to be due to the enhanced membrane fusion capability resulting from the increase in hydrophobicity of HEL and the affinity to lipopolysaccharides of the microbe. The HEL−GE conjugate prepared in this study with 1 mol of stearic acid/mol of HEL showed better emulsifying ability than the conjugate prepared by Ibrahim et al., as described above. However, in tests of the HEL−GE conjugate, antibacterial activity against Gram-negative bacteria such as *E. coli* was not observed. If glucose palmitic acid esters rather than stearic acid esters are conjugated with HEL, it is anticipated that the HEL−GE conjugate would exhibit an antimicrobial spectrum similar to that of the palmitic acid acylated HEL prepared by Ibrahim et al. (1991).

Concluding Remarks. In this study, we prepared a conjugate of HEL and glucose stearic acid monoester through the Maillard reaction and relatively little conformational change. The conjugate maintained ∼53−57% of the enzymatic activity of native HEL at 40−60 °C and exhibited considerable resistance to proteolysis. The emulsifying activity and emulsion stability were much enhanced, and the conjugate maintained ∼70% of the bactericidal activity of native HEL. This study demonstrates the possibility of preparing an antibacterial emulsifier by conjugating a fatty acylated saccharide with a protein through the Maillard reaction, without significant conformational changes in the protein moiety.

ABBREVIATIONS USED

HEL, hen egg lysozyme; GE, glucose stearic acid monoester (6-*O*-stearyl-D-glucose); NMR, nuclear magnetic resonance; TNBS, 2,4,6-trinitrobenzenesulfonate; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CD, circular dichroism; DSC, differential scanning calorimetry; T_o, onset temperature; T_p, peak temperature; T_c, conclusion temperature; EAI,

emulsifying activity index; IFO, Institute for Fermentation, Osaka.

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