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A Simple Method for the Preparation of an Amphiphilic Sequential Polypeptide

Masahiro Higuchi, Akira Takizawa, Takatoshi Kinoshita,* Yoshiharu Tsujita, and Kiyomi Okochi

Department of Materials Science and Engineering, Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya 466, Japan. *Received February 22, 1989; Revised Manuscript Received May 24, 1989*

ABSTRACT: A monolayer of poly(γ-methyl L-glutamate) (PMG) was prepared by placing a solution of PMG dissolved in chloroform at an air–water interface. The area occupied by the monolayer decreased to form the solid condensed state of the PMG monolayer. An aqueous solution of potassium hydroxide was added in large excess to the aqueous phase beneath the solid condensed monolayer, and as a result, the γ-methyl L-glutamate side chains hydrated in the aqueous phase were saponified. However, the residual side chains oriented into the air could not be hydrolyzed, yielding poly[(γ-methyl L-glutamate)-*co*-(potassium L-glutamate)]. The resulting polymer was washed with hydrochloric acid to produce an amphiphilic helical copolypeptide, poly[(γ-methyl L-glutamate)-*co*-(L-glutamic acid)] (am.-MG/GA). Hydrolysis of am.-MG/GA by Staphylococcus aureus, Strain V8 protease, and gel filtration chromatographic analysis determined that the sequence of am.-MG/GA forms an amphiphilic helix that is hydrophilic on one face (which consists of L-glutamic acid side chains) and hydrophobic on the opposite face (which comprises γ-methyl L-glutamate side chains). The amphiphilic character of am.-MG/GA was evident from the high solubility of the copolypeptide in both polar and apolar solvents.

Introduction

In the area of membrane biology, rapid progress has been made in understanding the primary and secondary structures of integral membrane proteins, such as ion channels[1–7] and several kinds of receptors.[8,9] Such studies gave rise to the idea that the transmembrane portions of many membrane proteins consist of bundles of parallel α-helices oriented perpendicular to the membrane surface. Furthermore, each helix is generally believed to contain both hydrophobic and hydrophilic amino acid side chains, periodically arranged so that all side chains facing the outside are hydrophobic, while those on the inside are hydrophilic.[8] Such amphiphilic molecules also provide effective reaction media, e.g., micelles[10] and reversed micelles,[11] which promote interfacial reactions. It seems, therefore, that studies of channel forming synthetic polypeptides may be important not only to the understanding of biological transmembrane channels but also to the development of a molecular basis for energy and/or information transfer systems and novel reaction media.

We are reporting on a novel and simple technique for the preparation of an amphiphilic sequential polypeptide whose α-helix is hydrophilic on one face and hydrophobic on the opposite face. The method used in this study involves the formation of a solid condensed monolayer of poly(γ-methyl L-glutamate) (PMG) at the air–water interface and the saponification of the PMG side chains hydrated in the aqueous phase, keeping the remaining side chains oriented away from the aqueous phase unreacted.

This method can easily be applied to other polypeptides carrying reactive side chains to produce unique α-helices having asymmetric character. Moreover, this method has the advantage that it permits simple and rapid production of such unique polypeptides regardless of their molecular weight, compared with traditional methods of synthesis of sequential polypeptides such as the activated ester method.[12,13]

Experimental Section

Materials. Poly(γ-methyl L-glutamate) (PMG). PMG was obtained by polymerization of the *N*-carboxy anhydride of L-glutamic acid γ-methyl ester (kindly provided by Ajinomoto Co., Ltd.) in 1,2-dichloroethane solution with *n*-hexylamine as an initiator.[14] The polymerization is as follows: the *N*-carboxy anhydride of L-glutamic acid γ-methyl ester (3.00 g; 0.0160 mol) was dissolved in freshly distilled 1,2-dichloroethane (100 mL). The solution was stirred and 5.43×10^{-4} mol of *n*-hexylamine (73 μL) added with stirring. The molar ratio of anhydride to initiator was 30. And then the solution was allowed to stand

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24 h at room temperature. The PMG obtained was precipitated in dry methanol. The intrinsic viscosity of PMG in dichloroacetic acid was determined by using an Ubbelohde viscometer at 25 °C. A molecular weight of 9000 was estimated from the intrinsic viscosity by the equation[15] $[\eta] = 2.24 \times 10^{-3} M^{0.58}$.

Random Copolypeptide Consisting of γ-Methyl L-Glutamate (MG) and L-Glutamic Acid (GA) (r-MG/GA). r-MG/GA was obtained as follows: the starting material, PMG, obtained in the previous section was dissolved in 1,2-dichloroethane (6.0 wt %, 10 mL). A mixed solution of methyl alcohol, isopropyl alcohol, and 0.4 M NaOH aqueous solution (2:2:1 volume ratio, 18 mL) was added to the solution with vigorous stirring at 20 °C. Under these reaction conditions, a homogeneous saponification of PMG was carried out for 10 h. The saponification of methyl ester side chains was confirmed by the high-resolution ^{1}H NMR spectra (Varian XL-200 spectrometer) of the product (r-MG/GA) in trifluoroacetic acid (10 mg/mL); i.e., the spectra showed the decrease in the peak at 3.9 ppm associated with side chain OCH_3 groups. From the degree of the decrease in the OCH_3 peak area, the L-glutamic acid content of the r-MG/GA was estimated to be 30 mol %.

Preparation of an Amphiphilic Sequential Polypeptide Consisting of γ-Methyl L-Glutamate and L-Glutamic Acid (am.-MG/GA). Our approach to prepare the amphiphilic sequential polypeptide, am.-MG/GA, is outlined in Figure 1. A known amount of poly(γ-methyl L-glutamate) (PMG) dissolved in chloroform was placed at the air–water interface in a hydrophobic vessel with a syringe. The solution spread over the interface and the solvent evaporated leaving the PMG monolayer. The area occupied by the monolayer was decreased by moving a hydrophobic barrier to form the solid condensed state of the monolayer. When the area per monomer residue of PMG reached 15 Å^2 (corresponding to the value denoted by an arrow on the surface pressure–area (π–A) isotherm of PMG in Figure 2), 40 mL of an aqueous solution of potassium hydroxide (KOH, 2.5 M) was injected into the aqueous phase (1000 mL; KOH, 0.1 M) beneath the solid condensed monolayer. The final molar ratio of KOH to γ-methyl L-glutamate residues was 10^5. After ca. 10 min, excess hydrochloric acid was added to convert the copolypeptide to its acidic form. The L-glutamic acid content of the resulting copolypeptide was estimated to be 34 mol % from an NMR analysis similar to that of r-MG/GA.

Methods

Surface Pressure–Area Isotherm of PMG. The surface pressure of the spread PMG monolayer was measured by the Wilhelmy technique using a glass plate attached to a sensitive torsion balance. The surface pressure was determined with a precision of $\pm 4 \times 10^{-5}$ N/m. A Teflon trough (0.16 m × 0.90 m × 0.01 m) was filled with doubly distilled water, and doubly distilled chloroform was used as a spreading solvent. Spreading of PMG on the water surface was carried out by applying 10 μL of a chloroform solution (0.5 mg/mL) of PMG with a Terumo microsyringe. Compression of the monolayer was carried out successively. The temperature of the substrate, 25 °C, was controlled to within 0.05 °C. Figure 2 shows a π–A isotherm for PMG obtained at 25 °C. The limiting area per monomer residue of PMG was 17.5 Å^2. The value calculated from X-ray diffraction measurements of α-helical PMG in solid films in which the molecules are packed hexagonally was shown to 17.9 Å^2.[16] It was confirmed, therefore, that the solid condensed PMG monolayer was compressed to form an orderly array of α-helices lying flat at the air–water interface. A similar result for the PMG monolayer has been reported by Shuler et al.[16]

Gel Filtration Chromatographic Analysis. Staphylococcus aureus, Strain V8 protease (V8 Protease), which preferentially hydrolyzed peptide bonds on the carboxyl-terminal side of glutamic acid,[17,18] was purchased from Seikagaku Kogyo Co., Ltd. The amphiphilic sequential copolypeptide (am.-MG/GA, 1 mg) and random copolypeptide (r-MG/GA, 1 mg) were incubated at 37 °C with 500 μg of V8 Protease in 50 mM sodium phosphate buffer solution, pH 7.8, for 24 h.[18] The total volume was 1 mL. After the reaction, the V8 Protease was removed by ultrafiltration (Molecut II LPG, Millipore Co., Ltd.). The identification of the oligopeptides produced by enzymic hydrolysis was performed as follows: The buffer solution, 20 μL, containing oligopeptides obtained was applied to a Wakopak WB-G-30 column (low–medium molecular weight range type, i.d. 7.8 mm × 300 mm, Wako-junyaku-kougyo Co. Ltd., Japan) for gel filtration chromatographic analysis with a refractive index detector (RI-3H, Japan Analytical Industry Co., Ltd.). The column was run upward at a flow rate of 0.5 mL/min (Model 655 Liquid Chromatograph, Hitachi Ltd.). The molecular weight of each oligopeptide fraction was determined by comparison with a calibration curve obtained by using different molecular weights of poly(ethylene oxides).

Solubility of Polypeptides in Aqueous Solution. The amphiphilic sequential copolypeptide (am.-MG/GA, 0.2 mg) and random copolypeptide (r-MG/GA, 0.2

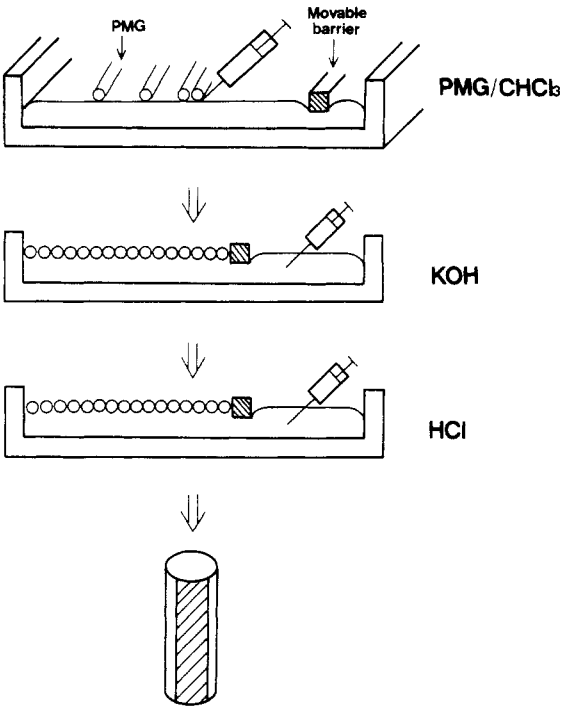


Figure 1. Preparation of an amphiphilic sequential polypeptide; shaded surface represents location of hydrophilic amino acid side chains and unshaded surface is hydrophobic.

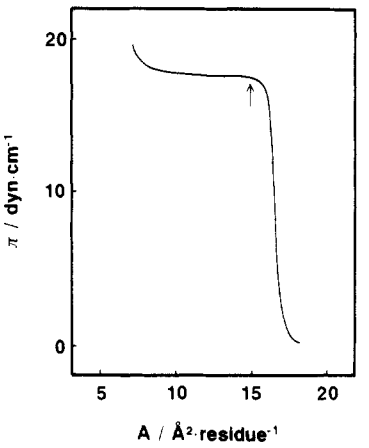


Figure 2. Pressure–area isotherm for poly(γ-methyl L-glutamate) at 25 °C.

mg) were incubated in a UV cell containing aqueous solutions at various pHs (3 mL), respectively. The aqueous solubility of the polypeptides was followed by measuring transmittance at 700 nm on a spectrophotometer (Jasco, UVDEC-670) at 25 °C, where the polypeptides have no absorption. As the measurements were carried out without stirring of the solution in the cell, an increase in the solubility of the polypeptide decreased the transmittance owing to scattering of light in the solution. Conversely, precipitation of the polypeptide increased the transmittance to the level of pure solvent.

Fluorescence Measurements. Quantitation of polypeptide molecules in solution was carried out by a fluorescence technique using 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD) as a probe. It is well-known that NBD, which itself is nonfluorescent, rapidly reacts with primary amino groups yielding a useful probe that fluoresces.[19] As the polypeptides used in this study had no primary amino groups in their side chains, NBD could be introduced at the amino-terminal end only. As a result, one molecule of the polypeptide was labeled with one NBD. A dimethylformamide solution (5 mL) containing the original PMG or saponified PMGs at a concentration of 1.4×10^{-5} residue mol/L was incubated at 60 °C with 10 μL of an ethanol solution of NBD (0.1 M) for 1 min. The strong fluorescence could be observed with the resulting solution containing the polypeptide carrying NBD. The excitation wavelength of NBD at the amino-terminal end of the polypeptides was 475 nm and the fluorescence was observed at 543 nm with a spectrofluorophotometer (Shimadzu Co. Ltd., FS-540) at 25 °C.

Circular Dichroism Measurements. CD spectra of PMG in trimethyl phosphate (TMP) and am.-MG/GA in TMP and aqueous solutions of pH 7.5, respectively, were measured with a Jasco, J-40, spectropolarimeter. The secondary structure was estimated from the molar ellipticity, $[\theta]_\lambda$. The concentration of polypeptide was fixed at 0.3 mg/10 mL.

Results and Discussion

Amino Acid Sequence Analysis. Despite the fact that a large excess of KOH was introduced into the aqueous phase beneath the PMG monolayer to saponify PMG (Figure 1), NMR analysis showed that the resulting copolypeptide contained only 34 mol % L-glutamic acid residues. When the same amount of KOH was applied to the saponify homogenously PMG dissolved in 1,2-dichloroethane, γ-methyl L-glutamate residues could be thoroughly converted to potassium L-glutamate along with some degradation of the polypeptide main chain. In order to clarify the possibility of peptide bond hydrolysis of PMG during the monolayer saponification, changes in the degree of polymerization after the reaction were estimated by a fluorescence technique. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD) was introduced at the amino-terminal ends of PMG (PMG*) and PMGs saponified under different monolayer conditions (saponified PMG*). It was confirmed that the fluorescence intensity of dimethylformamide solutions of PMG* and saponified PMG* was quantitatively proportional to the number of polypeptide chains per unit volume of the solvent.

Figure 3a shows the relationship between the ratio of the fluorescence intensity of saponified PMG* saponified for 10 min (I) to that of PMG* (I_{PMG}) and the area per monomer residue of the PMG monolayer at which the saponification was carried out. I/I_{PMG} values of more than unity at monolayer areas over ca. 16 Å²/residue imply that the cleavage of the PMG main chain occurred when the saponification was carried out. However, at an area

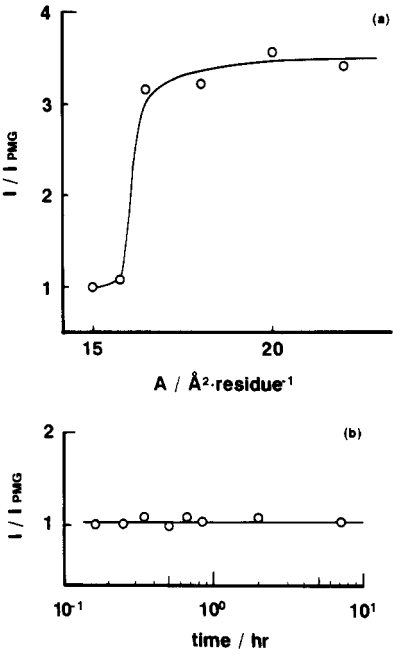


Figure 3. (a) Relationship between the relative fluorescence intensity of the saponified PMG*, saponified for 10 min, to that of PMG*, I/I_{PMG}, and the area per monomer residue of PMG. (b) Relationship between the relative fluorescence intensity, I/I_{PMG}, when the saponification was carried out at the area of 15 Å²/residue and the reaction time.

corresponding to a solid condensed monolayer, 15 Å²/residue, PMG escapes peptide bond cleavage during the saponification. It was also confirmed that PMG did not suffer peptide bond hydrolysis even over a period of 10 h when the saponification was carried out at the area of 15 Å²/residue (Figure 3b).

Very recently, Kawaguchi et al.[20] applied ellipsometry to investigate the behavior of polymer monolayers spread at the air–water interface. They showed that a monolayer thickness of PMG on the water surface is somewhat larger than those of other hydrophobic polymers such as poly(methyl methacrylate). The PMG monolayer, therefore, seems to be only slightly penetrating the aqueous phase. This result may explain the rather small extent of saponification, 34 mol %, of the copolypeptide obtained (am.-MG/GA). Saponification of 34 mol % of the total side chains suggests the possibility that the γ-methyl L-glutamate side chains within an angle of 120° (120°/360° = 1/3) on the cross-section of the α-helix rod (Figure 4) could be saponified along the helix axis. On the basis of Figure 4, a hypothetical sequence of am.-MG/GA can be written as

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
$$-(\mathrm{GA\,GA\,MG\,MG\,GA\,MG\,MG\,MG\,GA\,MG\,MG\,MG\,GA\,MG\,MG\,MG})_n- \quad (1)$$

where MG and GA denote γ-methyl L-glutamate and L-glutamic acid residues, respectively.

We attempted to determine the sequence of am.-MG/GA by enzymic hydrolysis of the polypeptide with V8 Protease. This enzyme specifically cleaves peptide bonds on the carboxyl-terminal side of glutamic acid.[16] Based on the hypothetical sequence in eq 1, the hydrolysis of am.-MG/GA by V8 Protease should yield unit sequences MGMGMGGA and MGMGGA and L-glutamic acid monomer, GA, as shown in eq 2.

Figure 5 shows (a) a gel filtration trace of am.-MG/GA hydrolyzed with V8 Protease and (b) a plot of the logarithm of the molecular weight of poly(ethylene oxides) against elution volume. The gel filtration trace had three

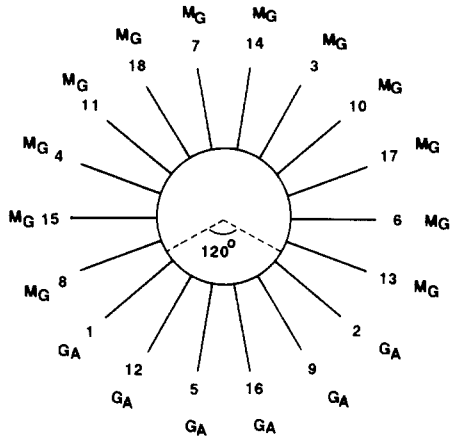


Figure 4. Top view of a schematic α-helix. MG, γ-methyl L-glutamate; GA, L-glutamic acid. The γ-methyl L-glutamate side chains within an angle of 120° were postulated to be converted to L-glutamic acid residues.

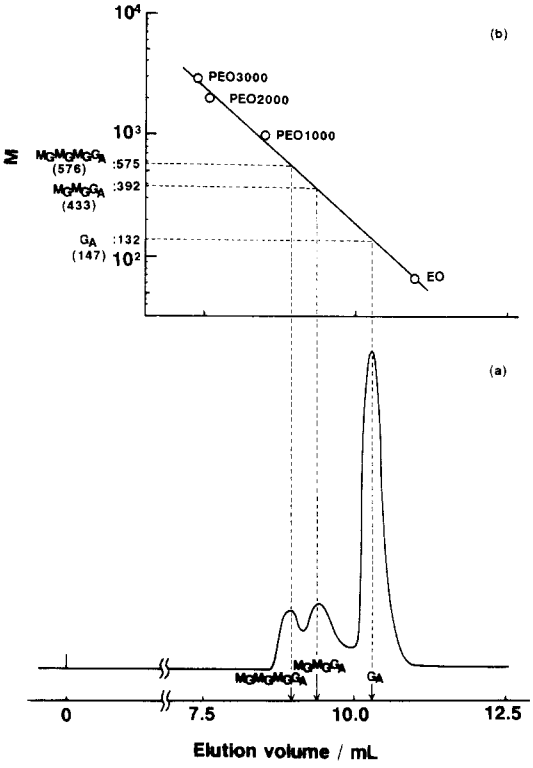
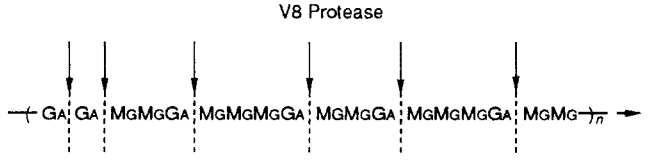


Figure 5. (a) Gel filtration chromatographic trace of the am.-MG/GA hydrolyzed with V8 protease. (b) Plot of the logarithm of the molecular weights of poly(ethylene oxides) against elution volume.



MGMGMGGA + MGMGGA + GA (2)

peaks indicating, as expected, that only three kinds of oligopeptides were generated in the hydrolysis of am.-MG/GA with V8 Protease. This result supports the possibility that am.-MG/GA has a sequence given by eq 1. Furthermore, the molecular weights of the oligopeptides in Figure 5(a) could be estimated to be 575, 392, and 132, respectively, from the calibration curve of poly(ethylene oxide) (Figure 5b). These molecular weights were rather close to those calculated for MGMGMGGA (576),

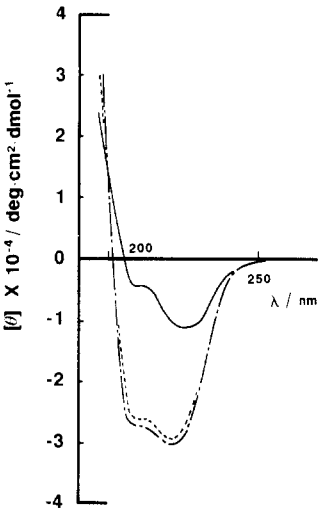


Figure 6. Circular dichroism spectra of polypeptide solutions: (- - -) PMG in TMP; (- · -) am.-MG/GA in TMP; (—) am.-MG/GA in aqueous solution at pH 7.5.

MGMGGA (433), and GA (147), respectively. On the other hand, the gel filtration trace of r-MG/GA (a random copolypeptide consisting of 30 mol % L-glutamic acid and 70 mol % γ-methyl L-glutamate) was quite different (the trace was not shown) from that of am.-MG/GA shown in Figure 5a. That is, the gel filtration for r-MG/GA showed broad peaks over a range of the molecular weight from 700 to 140. This means that the hydrolysis of r-MG/GA by V8 Protease yields a series of oligopeptides, $(MG)_nGA$ (n = 0–4), including luck components in the hydrolysis product of am.-MG/GA. The results, therefore, confirm that am.-MG/GA obtained by the saponification of PMG (Figure 1) has the sequence shown in eq 1.

Secondary Structure of Polypeptides. The CD spectra of PMG and am.-MG/GA are shown in Figure 6. CD spectra of trimethyl phosphate (TMP) solutions of PMG and am.-MG/GA showed that the two polypeptides exhibited similar values of $[\theta]_{222}$ associated with a stable right-handed α-helix in TMP. This suggests that racemization did not occur during the saponification. Furthermore, am.-MG/GA has an α-helical conformation even in an aqueous solution at pH 7.5. The helix content estimated was 38%. Epand et al. have reported[21] that amphiphilic polypeptides, whose helix content is ca. 40%, self-associate in an aqueous solution. It was also confirmed that r-MG/GA was not soluble in an aqueous solution (vide infra). These results suggest, therefore, that am.-MG/GA has an amphiphilic α-helical structure, which results in an increase in the solubility of the polypeptide.

Solubility of Polypeptides. Figure 7 shows the pH dependence of the transmittance of aqueous solutions containing am.-MG/GA and r-MG/GA. Since all solution of r-MG/GA precipitated at the bottom of the UV cell, the transmittance of the aqueous solution was almost the same as that of pure water over the pH range from 4.0 to 8.5. The insolubility of r-MG/GA is attributable to a large number of hydrophobic MG residues.

The transmittance of the aqueous am.-MG/GA solution, however, decreased above pH 6.3, owing to an increase in the solubility of am.-MG/GA (scattering of light) in the aqueous solution above neutral pH, even though the content of the hydrophobic MG residue of am.-MG/GA is almost the same as that of r-MG/GA.

To understand the mechanism of dissolution of am.-MG/GA into water, the temperature dependency of the

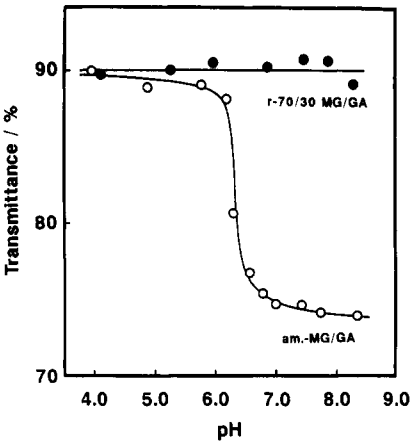


Figure 7. pH dependence of the transmittance of aqueous solutions containing am.-MG/GA and r-70/30 MG/GA.

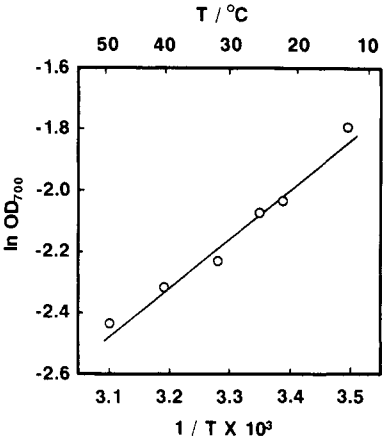


Figure 8. Temperature dependence of the optical density of am.-MG/GA in aqueous solution at pH 7.5.

optical density of am.-MG/GA in an aqueous solution was studied at pH 7.5 (Figure 8). The logarithm of the optical density of an aqueous solution containing am.-MG/GA decreased with increasing temperature, indicating that the dissolution of am.-MG/GA into water is exothermic. The value of ΔH was estimated to be −3 kcal/mol. This result implies that am.-MG/GA takes an energetically favorable structure in the aqueous phase, similar to surface-active agents and/or "polysoaps", to avoid the polar–apolar interactions between water and the hydrophobic side chains of am.-MG/GA. In addition, am.-MG/GA was highly soluble in chloroform, dimethylformamide, and trimethyl phosphate, thus confirming the amphiphilic character of am.-MG/GA.

These results are consistent with the hypothesis that am.-MG/GA has a unique sequence given by eq 1 and thus forms an amphiphilic α-helix, i.e., a helix that is polar on one face and apolar on the opposite face as is shown in Figures 1 and 4.

The channel-forming activity of am.-MG/GA in a lipid bilayer membrane is under investigation.

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Registry No. PMG (homopolymer), 25086-16-2; PMG (SRU), 25036-43-5; r-MG/GA, 66844-09-5; L-glutamic acid γ-methyl ester *N*-carboxy anhydride, 1663-47-4; protease, 9001-92-7.