JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Microstructure of Poly(γ-glutamic acid) Produced by *Bacillus subtilis* Consisting of Clusters of D- and L-Glutamic Acid Repeating Units

FEI WANG,[†] MASAJI ISHIGURO,[†] MAI MUTSUKADO,[‡] KEN-ICHI FUJITA,[‡] AND TOSHIO TANAKA*[,‡]

Suntory Institute for Bioorganic Research, 1-1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618-0024, Japan, Graduate School of Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan

Poly(γ-glutamic acid) (PGA) produced by a strain of *Bacillus subtilis* was partially hydrolyzed into various oligopeptides so that the dipeptide fraction was isolated by the preparative thin-layer chromatography. HPLC analysis was applied to the detection of each of the four stereoisomers in this fraction using chemically synthesized authentic samples. The fraction consisted of *N*-γ-D-glutamyl-D-glutamic acid, *N*-γ-L-glutamyl-L-glutamic acid, *N*-γ-D-glutamyl-L-glutamic acid, and *N*-γ-L-glutamyl-D-glutamic acid at a ratio of 5.9:6.0:1.0:1.0. On the basis of this result, a model was proposed for the microstructure of the bacterial PGA, in which D- and L-glutamic acid repeating units are alternately linked in a single chain of the molecule.

KEYWORDS: Poly(γ-glutamic acid); *Bacillus subtilis*; microstructure

INTRODUCTION

Poly(γ-glutamic acid) (PGA) is an extremely viscous polypeptide of glutamic acid, which elongates via the amide linkage between the γ-carboxyl and amino groups (*1*). Both D- and L-glutamic acid stereoisomers are commonly detected in the acid hydrolysate of PGA produced by various strains of *Bacillus subtilis* (*2, 3*). On the other hand, D-glutamic acid is predominantly detected in the hydrolysate of PGA produced by *Bacillus licheniformis* and *Bacillus anthracis* (*4, 5*), whereas L-glutamic acid is a major component of PGA produced by the alkalophilic strain of *Bacillus* sp (*6*). More recent studies revealed the polymerization of L-glutamic acid alone in PGAs from a eukaryotic organism such as *Hydra* and a strain of an extremely halophilic *Arcahaeon* (*7, 8*). These findings suggest the preferential polymerization of either D- or L-glutamic acid residues by PGA synthesizing enzyme with a strict specificity toward either of these stereoisomers. We analyzed the microstructure of PGA produced by *B. subtilis* F-2-01 with the aid of PGA hydrolase that specifically hydrolyzes the linkage between L-glutamic acid residues. A model was thus proposed for the microstructure of the bacterial PGA, in which D- and L-glutamic acids are copolymerized in a single chain of the molecule (*9*). This model was later supported by the ^{13}C NMR analysis of poly(α-ethlyl γ-D,L-glutamate), which was prepared from PGA, as well as the stereospecificity of the PGA synthetic system-associated cell membrane of *B. subtilis* in the polymerization of both D- and L-glutamic acids (*10, 11*). The existence of such a stereochemically heterogeneous peptide unit in PGA should be more directly demonstrated by the detection of a covalently bound dipeptide unit of D- and L-glutamic acids in a linear chain of PGA.

Recent progress in peptide synthetic chemistry has enabled the syntheses of *N*-γ-D-glutamyl-L-glutamic acid (D-L) and *N*-γ-L-glutamyl-D-glutamic acid (L-D). We therefore attempt to detect such stereochemically heterogeneous dipeptides in the partial acid hydrolysate of PGA by HPLC using chemically synthesized dipeptides as their authentic markers. A model is proposed for the microstructure of the bacterial PGA on the basis of the ratio of four stereoisomers constituting the dipeptide fraction.

MATERIALS AND METHODS

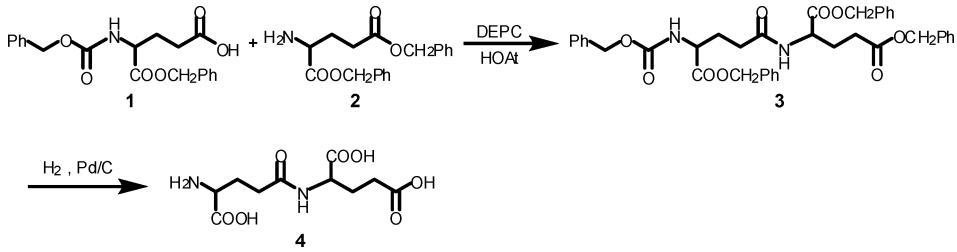
Materials. Diethyl phosphorocyanidate (DEPC), 1-hydroxy-7-azabenzotriazole hydrochloride (HOAt), and the derivatives of D- and L-glutamic acid (**1**, **2**) were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). Pd/C (20 wt %), citric acid, and sodium bicarbonate were purchased from Nacalai Tesque Inc. (Kyoto, Japan). PGA of *B. subtilis* F-2-01 is a product of Yakult Yakuhin Co. (Tokyo, Japan), formerly manufactured by Meiji Seika Kaisha Co. (Tokyo, Japan). Unless otherwise indicated, all commercially available reagents were used without further purification.

NMR and MS. ^1H and ^{13}C NMR spectra were recorded respectively at 400 and 100 MHz in $CDCl_3$ and D_2O on a JEOL JNM-EX400 spectrometer. Chemical shifts are recorded as δ values in parts per million (ppm). Coupling constants (*J*) are reported in hertz (Hz). The abbreviations of multiplicity are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad. Infrared spectra were

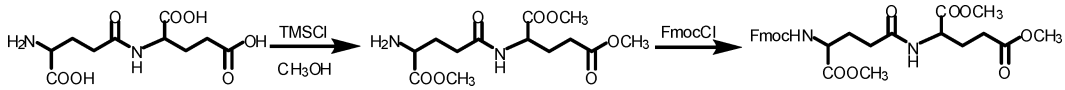
* To whom correspondence should be addressed. Tel: 81-6-6605-3163. Fax: 81-6-6605-3164. E-mail:tanakato@sci.osaka-cu.ac.jp
[†] Suntory Institute for Bioorganic Research.
[‡] Osaka City University.

10.1021/jf8001262 CCC: $40.75
Published on Web 05/20/2008

Scheme 1. Syntheses of Four Stereochemical Isomers of Glutamic Acid Dimer



Scheme 2. Syntheses of F-moc Derivatives of D-D, L-L, D-L, and L-D



recorded on a Shimadzu IRPrestige-21 spectrometer. The absorption bands of IR spectra are in cm^{-1}. High-resolution mass spectra (HRMS) were acquired using a Shimadzu LCMS-IT-TOF mass spectrometer.

Syntheses of F-moc Derivatives of *N*-γ-D-Glutamyl-D-glutamic acid (D-D), *N*-γ-L-Glutamyl-L-glutamic acid (L-L), D-L, and L-D. *Syntheses of Four Dimers of Glutamic Acid (***Scheme 1***).* To a solution of **1** (111.4 mg, 0.3 mmol) and **2** (98.2 mg, 0.3 mmol) in anhydrous dichloromethane were added diethyl phosphorocyanidate (DEPC) (58.7 mg, 0.36 mmol) and 1-hydroxy-7-azabenzotriazole hydrochloride (HOAt) (44.9 mg, 0.33 mmol) at 0 °C. The resulting mixture was stirred at room temperature overnight. The reaction mixture was diluted with ethyl acetate, and the organic layer was washed with 10% citric acid (50 mL × 2), saturated sodium bicarbonate (50 mL × 2), and brine (50 mL); dried over anhydrous magnesium sulfate; and concentrated under vacuum. Flash chromatography afforded **3** in 93−96% yield. *N*-(*O*-Benzyl-*N*-benzyloxycarbonyl-D-γ-glutamyl)-D-glutamic acid dibenzyl ester: Yield = 192 mg (94%). ^{1}H NMR (400 MHz, δ ppm, $CDCl_3$): 1.92−2.03 (2H, m, C*H*$_2$CH), 2.19 (4H, br, C*H*$_2$COO), 2.29−2.44 (2H, m, C*H*$_2$CH), 4.38 (1H, br, C*H*), 4.60 (1H, br, C*H*), 5.03−5.17 (8H, m, C*H*$_2$Ph), 5.62 (1H, br d, J = 7.2 Hz, N*H*), 6.31 (1H, br d, J = 6.4 Hz, N*H*), 7.12−7.35 (20H, m, Ar-*H*). ^{13}C NMR (100 MHz, δ ppm, $CDCl_3$): 27.19, 28.20, 30.23, 31.99, 51.82, 53.51, 66.52, 67.06, 67.30, 67.32, 127.99 (d), 128.06 (d), 128.15 (d), 128.20 (d), 128.21 (d), 128.27 (d), 128.41 (t), 128.48 (d),128.55 (t), 135.06, 135.12, 135.62, 136.10, 156.04, 171.42, 171.46, 171.56, 172.43. IR (cm^{-1}): 3315 (NH), 2960 (CH), 1732 (ester C═O), 1690 (amide C═O), 1647 (amide C═O), 1541 (NH), 1449, 1389, 1275, 1171, 1059, 960. HRMS (*m*/*z*): calcd. for $C_{39}H_{40}N_2NaO_9$ ($M + Na^+$) 703.2626, found 703.2640. The NMR and HRMS spectral data were identical or fully comparable to those detected with each of *N*-(*O*-benzyl-*N*-benzyloxycarbonyl-L-γ-glutamyl)-L-glutamic acid dibenzyl ester, *N*-(*O*-benzyl-*N*-benzyloxycarbonyl-D-γ-glutamyl)-L-glutamic acid dibenzyl ester, and *N*-(*O*-benzyl-*N*-benzyloxycarbonyl-L-γ-glutamyl)-D-glutamic acid dibenzyl ester.

A mixture of **3** (100 mg, 0.147 mmol) and Pd/C (20 wt %, 50 mg) in 95% ethanol was stirred at room temperature for 24 h under hydrogen atmosphere. The reaction mixture was filtered, and the filtrate was concentrated under vacuum. The residue was triturated with methanol/$CHCl_3$ to precipitate white powder, then the white powder was dissolved in water and freeze-dried after filtration to give **4** in 64−71% yield. D-D: Yield = 26 mg (64%). ^{1}H NMR (400 MHz, δ ppm, D_2O): 1.83−1.93 (1H, m, CHC*H*$_2$), 1.97−2.13 (3H, m, CHC*H*$_2$), 2.35 − 2.48 (4H, m, C*H*$_2$COO), 3.70 (1H, t, J = 6.8 Hz, NH$_2$C*H*CH$_2$), 4.22 (^{1}H, dd, J = 4.8 Hz, 8.8 Hz, NHC*H*CH$_2$). ^{13}C NMR (100 MHz, δ ppm, D_2O): 26.98, 27.07, 31.28, 32.24, 54.03, 54.84, 174.38, 175.13, 177.14, 178.17; IR (cm^{-1}): 3325 (NH, COOH), 2947 (CH), 1717 (C═O), 1636 (C═O), 1558 (NH), 1406, 1250, 1080. HRMS (*m*/*z*): calcd for $C_{10}H_{17}N_2O_7$ ($M + H^+$) 277.1030, found 277.1033. The NMR and HRMS spectral data were identical or fully comparable to those detected with each of L-L, D-L, and L-D.

*Syntheses of F-moc Derivatives (***Scheme 2***).* To the sample in anhydrous methanol was added 0.5 mL of trimethylchlorosilane (TMSCl), and the resulting mixture was stirred for 3 h. After evaporating the solvent of the reaction mixture under vacuum, the residue was dissolved in 5 mL of dioxane:water (1:1), and then 9-fluorenylmethylcarbonyl chloride (F-moc Cl) (3.5 mg, 0.0135 mmol) and $NaHCO_3$ (22.8 mg, 0.271 mmol) were added. The reaction mixture was stirred overnight. After removing the solvent under vacuum, the residue was dissolved in 3 mL of hexane:2-propanol (75:25) and the solution was filtered using a 0.45 μm membrane for HPLC.

HPLC Separation of F-moc Derivatives of D-D, L-L, D-L, and L-D. HPLC was performed using a Waters 2690 HPLC system equipped with a photodiode array detector and a scanning fluorescence detector, in which F-moc derivatives were separated by a Chiralcel IA column (4.6 × 250 mm) at 20 °C and were detected with excitation at 250 nm and emission at 335 nm. Isocratic elution was performed using a mixture of hexane and 2-propanol (75:25) as the mobile phase at a flow rate of 0.7 mL/min. The standard F-moc derivatives were fully separated under the above analytical conditions, in which the derivatives of D-D, D-L, L-D, and L-L were eluted at the retention times of 25.1, 28.9, 31.2, and 35.0 min, respectively. Their peak areas were proportional to the quantities injected into the column in the range of 10−200 pmol with the correlation coefficients of more than 0.999. The relative standard deviation was kept less than 3.1% of the average quantity in three different experiments for the measurement of each derivative.

Preparation of Dipeptide Fraction from PGA. PGA of *B. subtilis* F-2-01 (50 mg) was dissolved in 5 mL of 1.0 N HCl and partially hydrolyzed to yield various oligopeptides at 100 °C for 2.5 h. The hydrolyzed sample was then evaporated to dryness and the resulting pellet was dissolved in distilled water. This procedure was repeated several times for the complete removal of HCl. Oligopeptides were separated by preparative thin-layer chromatography using a silica gel plate (Kieselgel 60, Merck) and a mixture of acetic acid, butanol, and water (1:1:1) as the solvent. The silica gel layers containing each of the glutamic acid, dipeptide, and tripeptide fractions were removed from the plate, and these components were extracted with distilled water. The extracts were appropriately concentrated and their purity was analyzed by thin-layer chromatography. As shown in **Figure 1**, the spots of glutamic acid, dipeptides, and tripeptides were fully separated

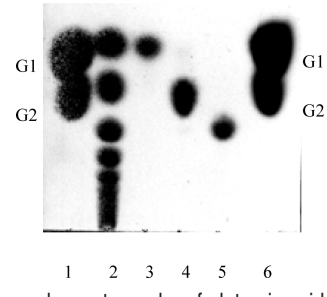


Figure 1. Thin-layer chromatography of glutamic acid (lane 3), dipeptide (lane 4), and tripeptide (lane 5) from acid hydrolysate of PGA (lane 2). Each sample was spotted onto a silica gel plate (Kieselgel 60, Merck) and developed together with L-glutamic acid (G1) and L-L (G2) (lane 1 and 6) as markers. The plate was sprayed with a ninhydrin reagent and heated at 100 °C to visualize each spot.

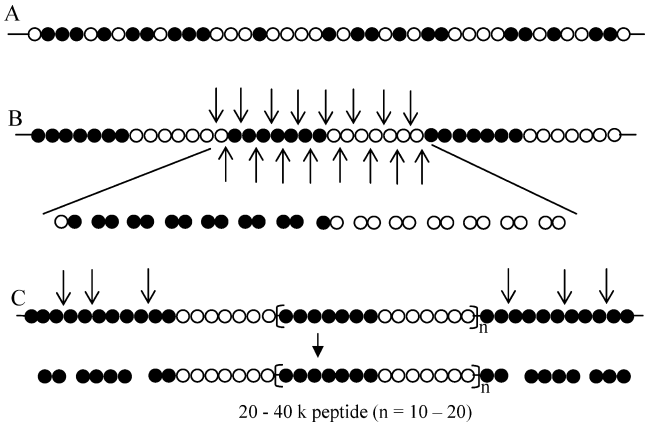


Figure 2. Proposed microstructures of PGA deduced from the ratio of D-D, L-L, D-L, and L-D. (**A**) D- (○) And L- (●) glutamic acid residues are randomly linked at a ratio of 1.0:1.0 in a single chain of PGA, in which D-D, L-L, D-L, and L-D are arranged at an approximate ratio of 1.0:1.0:1.0:1.0. (**B**) D- (○) And L- (●) glutamic acids are linked in a linear chain of PGA at a ratio of 1.0:1.0, in which D-D, L-L, D-L, and L-D are arranged at an approximate ratio of 6.0:6.0:1.0:1.0. The arrows indicate every combination of the sites for the complete hydrolysis of PGA into dipeptides. (**C**) The mode of production of a 20K−40K peptide by the action of PGA hydrolase is expected on the assumption that the enzyme cannot split the regions where L-glutamic acid residues are sequentially linked to a unit with the size of a heptapeptide. The arrows indicate possible hydrolysis sites of PGA hydrolase.

from other components so that these extracted samples could be used in the analysis of their compositions in terms of D- and L-glutamic acid.

The mixture of synthetic dipeptide stereoisomers was heated in 1.0 N HCl at 100 °C for 2.5 h and analyzed for their contents by HPLC. These stereoiomers were detected at the ratios with no significant differences before and after hydrolysis. This indicates that the ratio of stereoisomers in the dipeptide fraction from PGA primarily depends on their mode of arrangements in the molecule.

RESULTS AND DISCUSSION

Glutamic acid isolated from the partial acid hydrolysate of PGA was analyzed for its D- and L-glutamic acid contents by HPLC according to our previously described method (*9*). D- And L-glutamic acids were detected at a ratio of 1.0:1.0. The dipeptide fraction was found to consist of D-D, L-L, D-L, and L-D at a ratio of 5.9:6.0:1.0:1.0, and the overall ratio of D- and L-glutamic acids therein agreed with a ratio of D- and L-glutamic acids (1.0:1.0) obtained above. This study demonstrates the copolymerization of D- and L-glutamic acid residues in a single chain of PGA on the basis of the detection of D-L and L-D. The ratio of D- to L-glutamic acid in PGA may represent equal stereospecificities of glutamic acid ligase of the PGA synthesizing enzyme toward D- and L-glutamic acids as substrates for polymerization.

Figure 2A shows a structure of PGA in which D- and L-glutamic acid residues are randomly linked at a ratio of 1.0:1.0 so that D-D, L-L, D-L, and L-D are available at an approximate ratio of 1.0:1.0:1.0:1.0. Differently from this structure, however, the experimental data on the ratio of these stereoisomers indicate the existence of D- and L-glutamic acid repeating units, which are alternately linked in a linear chain of PGA. We thus propose a model of the structure of the bacterial PGA (**Figure 2B**) in which the average numbers of D- and L-glutamic acid repeating units are deduced to be seven from experimental data (5.9:6.0:1.0:1.0). This means that the number of glutamic acid residues in each of the repeating units can be larger than seven in few regions of PGA. Martínez de Ilarduya et al. analyzed the microstructure of poly(α-ethyl γ-glutamate) obtained from PGA with the aid of ^{13}C NMR and for the first time revealed its existence as the racemic stereocopolymers together with a minor amount of a mixture of the two enantiomerically pure homopolymers (*10*). In agreement with our results, stereoblocks with a number-average sequence length of 7−11 enantiomeric units were found to be the most probable sequences existing in biosynthetic PGA.

In our previous study, the bacterial PGA was converted into a peptide (20K−40K) in which D- and L-glutamnic acid residues are commonly detected, with the accompanying production of dipeptides, tripeptides, and tetrapeptides of L-glutamic acid alone by the action of PGA hydrolase with a strict stereospecificity toward L-glutamic acid (*9*). This peptide was found to contain two or three L-glutamic acid residues at both N- and C-terminals of the molecule. This result suggests that the enzyme can split the bacterial PGA in the region where L-glutamic acid residues are sequentially linked to a heptapeptide unit. As shown in **Figure 2C**, however, the heptapeptide unit seems to be too short to be the site of the enzymatic hydrolysis, since this unit most frequently appears in the structure of PGA (**Figure 2B**) and its enzymatic hydrolysis should degrade PGA to peptides much smaller than the experimentally obtained peptide (20K−40K). The region susceptible to the enzyme action should have a cluster of L-glutamic acid repeating units with a more increased size that are rarely distributed in the linear chain of PGA. However, it remains to be solved how D- and L-glutamic acid residues sequentially elongate to form clusters and how these clusters are alternately linked in a linear chain of the bacterial PGA. There seems to be a clue to the mode of polymerization of glutamic acid residues by PGA synthesizing enzyme in the relationship between the spatial structure of the growing PGA molecule and the stereochemical conformation of glutamic acid isomers incorporated into this growing chain of PGA.

ABBREVIATIONS USED

PGA, poly(γ-glutamic acid); D-D, *N*-D-γ-glutamyl-D-glutamic acid; L-L, *N*-L-γ-glutamyl-L-glutamic acid; D-L, *N*-D-γ-glutamyl-L-glutamic acid; L-D, *N*-L-γ-glutamyl-D-glutamic acid; HRMS, high-resolution mass spectra; DEPC, diethyl phosphorocyanidate; HOAt, 1-hydroxy-7-azabenzotriazole hydrochloride; TMSCl, trimethylchlorosilane.

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Received for review January 15, 2008. Revised manuscript received March 25, 2008. Accepted March 28, 2008.

JF8001262