Supporting Information

Involvement of peptide epimerization in poly-γ-glutamic acid biosynthesis

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1. Methods

1-1. General

All chemicals were purchased from Sigma-Aldrich Japan (Tokyo, Japan), Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), or Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). PGA standards were purchased from Sigma-Aldrich Japan (mol wt 3,000–15,000, 15,000–50,000, and 50,000–100,000) and Fujifilm Wako Pure Chemical Corporation (mol wt 200,000–500,000). Oligo nucleotide was obtained from Fasmac (Kanagawa, Japan). Enzymes, molecular weight standards and kits for DNA manipulation were purchased from Takara Bio (Shiga, Japan), or New England Biolabs Japan (Tokyo, Japan). PCR reactions were carried out using a GeneAmp PCR System 9700 thermal cycler with Tks Gflex DNA polymerase (Takara Bio). DNA sequences were determined by a commercial company (Fasmac). General genetic manipulations of *E. coli* were performed according to standard protocols.[1] NMR spectra were obtained using a JEOL ECS-400 spectrometer, and chemical shifts (δ in parts per million) are reported relative to that of the solvent peak (δ = 4.65 for D_2O in ^1H NMR spectra). Amino acid analysis was performed using post column ninhydrin derivatization method with Hitachi High-Speed Amino Acid Analyzer Model L-8900 at the Open Facility, Global Facility Center, Creative Research Institution, Hokkaido University. Amino acids were monitored at 440 nm and 570 nm. PGA in agarose gels was visualized with methylene blue solution containing 0.5% (w/v) methylene blue and 3% (v/v) acetic acid.

1-2. Bacterial strains and cultures

Bacillus subtilis 168 and *Bacillus megaterium* NBRC 15308 was obtained from NITE Biological Resource Center, the National Institute of Technology and Evaluation (Tokyo, Japan) and cultivated according to the supplier's protocol. *E. coli* WM335 was kindly provided by Professor Walter Messer of the Max-Planck Institute for Molecular Genetics and cultivated in the LB Broth (Lennox) supplied with 100 μg/mL D-glutamic acid.

1-3. Expression of *pgsBCA* in *E. coli* WM335

The *pgsBCA* was amplified by PCR using *B. megaterium* NBRC 15308 genomic DNA using a primer pair BM_pgsBCA_F: 5′-GATGCCATGGTCTTAATTATCCTATGCTGCCTATTTTTAGTAGGG-3′ and BM_pgsBCA_R: 5′-GATGGATCCTTAGTTAGCTTGCGCTTCGTTCTTTTTGCGCTCCTC-3′. After restriction enzyme digestion with *Nco*I and *Bam*HI (underlined), the DNA fragment was cloned into the same sites of the vector pTrc99A and the obtained plasmids were introduced into *E. coli* WM335 to yield *E. coli* WM335/pTrc99A-pgsBCA. A liquid culture of the transformant grown at 37°C in the production medium (LB broth supplied with 0.5% $MgCl_2 \cdot 6H_2O$, 0.05% $MnSO_4 \cdot 5H_2O$, 60 μg/mL ampicillin, and 100 μg/mL D-Glu) was induced by adding 1 mM IPTG when the optical density at 600 nm reached about 1. The cultivation was continued for an additional 24 hours at 28°C. L-[4,4-$^2H_2$]glutamic acid hydrochloride was added to the production medium for the isotope tracer experiments with labeled L-Glu, and D-[4,4-$^2H_2$]glutamic acid hydrochloride was supplied instead of non-labeled D-Glu.

1-4. Purification of PGA

The culture (200 mL) was centrifuged at 8,000×*g* for 20 min and the cell cake was washed twice with 0.14 M NaCl (20 mL each). The culture supernatant and the wash solution were combined, adjusted pH to 2.5 with 6 M sulfuric acid, and left to stand 2 hours at 4°C to precipitate polysaccharides.[2] After removal of the precipitate by centrifugation at 8,000×*g* for 10 min, the supernatant was mixed with 3 volumes of cold absolute ethanol, followed by centrifugation at 4,000×*g* for 20 min. The resulting pellet was then dissolved in 2 mL distilled water and dialyzed three times against distilled water at ambient temperature. The dialyzed solution was lyophilized, redissolved in 2

mL distilled water, and centrifuged at 20,000×g for 1 h. The supernatant was treated with Proteinase K (40 μg/mL) at 37°C for overnight, and then dialyzed three times against distilled water at ambient temperature. The sample was again lyophilized, redissolved in 150 μL gel loading buffer (mixture of 100 μL water and 50 μL NEB Gel Loading Dye, Purple, 6X), and purified by agarose gel electrophoresis with the following conditions: 1.6% agarose gel, 1×TAE running buffer, 100 V. A small portion of the gel was stained with methylene blue to visualize PGA and a gel slice containing PGA was excised from the remaining unstained gel. Finally, PGA was recovered from the gel slice by electroelution and the buffer was exchanged to prepare ca. 10% PGA solution in 0.1 M Tris-HCl (pH 8.0) by ultrafiltration (Amicon Ultracel-30, 0.5 mL, Merck). PGA samples were stored at −30°C until analysis. For NMR analysis, 10 mM ammonium formate solution was used for the ultrafiltration and the resulting PGA solution was lyophilized.

1-5. Preparation of recombinant enzymes, YoqZ and GGT

The *yoqZ* was amplified by PCR using *B. subtilis* 168 genomic DNA as a template and the following primers. Bsub_yoqZ_F(NdeI): 5′-GGAATTGCATATGCTAGCTGCAGATAAGTATAG-3′ and Bsub_yoqZ_R(XhoI): 5′-AATCCTCGAGTGGTGCTACACCAGCCATACAATAC-3′. The PCR product was cloned into the *Nde*I-*Xho*I site of pET28b vector and the resulting plasmid was introduced into *E. coli* BL21(DE3). The expression and purification of YoqZ were accomplished based on a reported procedure.[3] Purified YoqZ (24.3 kDa) was analyzed by SDS-PAGE on 10% gels. The proteins were visualized by Coomassie brilliant blue staining. (Figure S4) Because the *ggt* of *B. subtilis* 168 contains one *Nde*I site, we introduced a silent mutation to remove the *Nde*I site by overlap extension PCR. The N-terminal and C-terminal regions of *ggt* were amplified by PCR using two primer sets, Bsub_GGT_F(NdeI): 5′-GGAATTGCATATGGATGAGTACAAACAAGTAGATG-3′/Bsub_GGT_in_R: GACGCACGGTCGGCATAGGACAAATGCATCGTTTC-3′ and Bsub_GGT_in_F: 5′-GAAACGATGCATTTGTCCTATGCCGACCGTGCGTC/Bsub_GGT_C(BamHI): 5′-AATCGGATCCTCGAGCTCATTTACGTTTTAAATTAATGCCGAT-3′, respectively. Two PCR products were then mixed, treated with *Dpn*I to remove the genomic DNA, and used as the template for PCR with primer pair of Bsub_GGT_F(NdeI) and Bsub_GGT_C(BamHI). The PCR product was cloned into the *Nde*I-*Bam*HI site of pCold-I vector and the resulting plasmid was introduced into *E. coli* BL21(DE3). After expression of GGT using the methods in previous report, His-tagged GGT was purified with Ni-NTA resin (Qiagen). As shown in Figure S4, purified GGT is composed of the large (42.5 kDa) and small (20.0 kDa) subunits due to autocatalytic posttranslational processing of full length GGT (62.5 kDa).[4, 5]

1-6. Hydrolysis of PGA and LC-MS analysis

The PGA was hydrolyzed using YoqZ and GGT. The reaction mixture (16 μL) containing 10 μL PGA, 6 μM YoqZ, 11 μM GGT, and 100 μM $ZnSO_4$ in 60 mM Tris-HCl (pH 8.0) was incubated for 2 h at 37°C. The resulting solution containing D/L-Glu was directly used for N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (L-FDAA) derivatization by adding 5 μL of 1 M sodium bicarbonate and 25 μL of 1% L-FDAA dissolved in acetone. The reaction mixture was incubated at 37°C for 1 h. After the reaction was quenched by the addition of 5 μL of 1 M HCl, the reaction mixture was diluted with 200 μL methanol and 250 μL water. After centrifugation, the supernatant containing L-FDAA derivatives was analyzed by LC-ESI-MS under the following conditions: Waters ACQUITY UPLC system equipped with a Photodiode Array Detector and a SQ Detector2; Mightysil RP-18 GP Aqua column (150 mm × 2.0 mm, 3 μm, Kanto Chemical Co., Inc., Tokyo, Japan); flow rate, 0.2 mL/min; temperature, 40°C; mobile phase A, water containing 0.05 vol% trifluoroacetic acid, mobile phase B, methanol containing 0.05 vol% trifluoroacetic acid; gradient conditions, 25% B, 0–2 min; 25–80% B, 2–20 min; detection, ESI negative ion mode; injection volume, 10 μL.

1-7. Synthesis of L- and D-[4,4-$^2H_2$]glutamic acid.

L-[4,4-$^2H_2$]Glutamic acid was prepared using previously reported procedure.[6] D-[4,4-$^2H_2$]Glutamic was also prepared using same method. No racemization was detected during the reaction as shown in Figure S7. The ^{1}H-NMR spectra were shown in Figure S8 (>95% ^{2}H for both products).

2. Supplemental figures

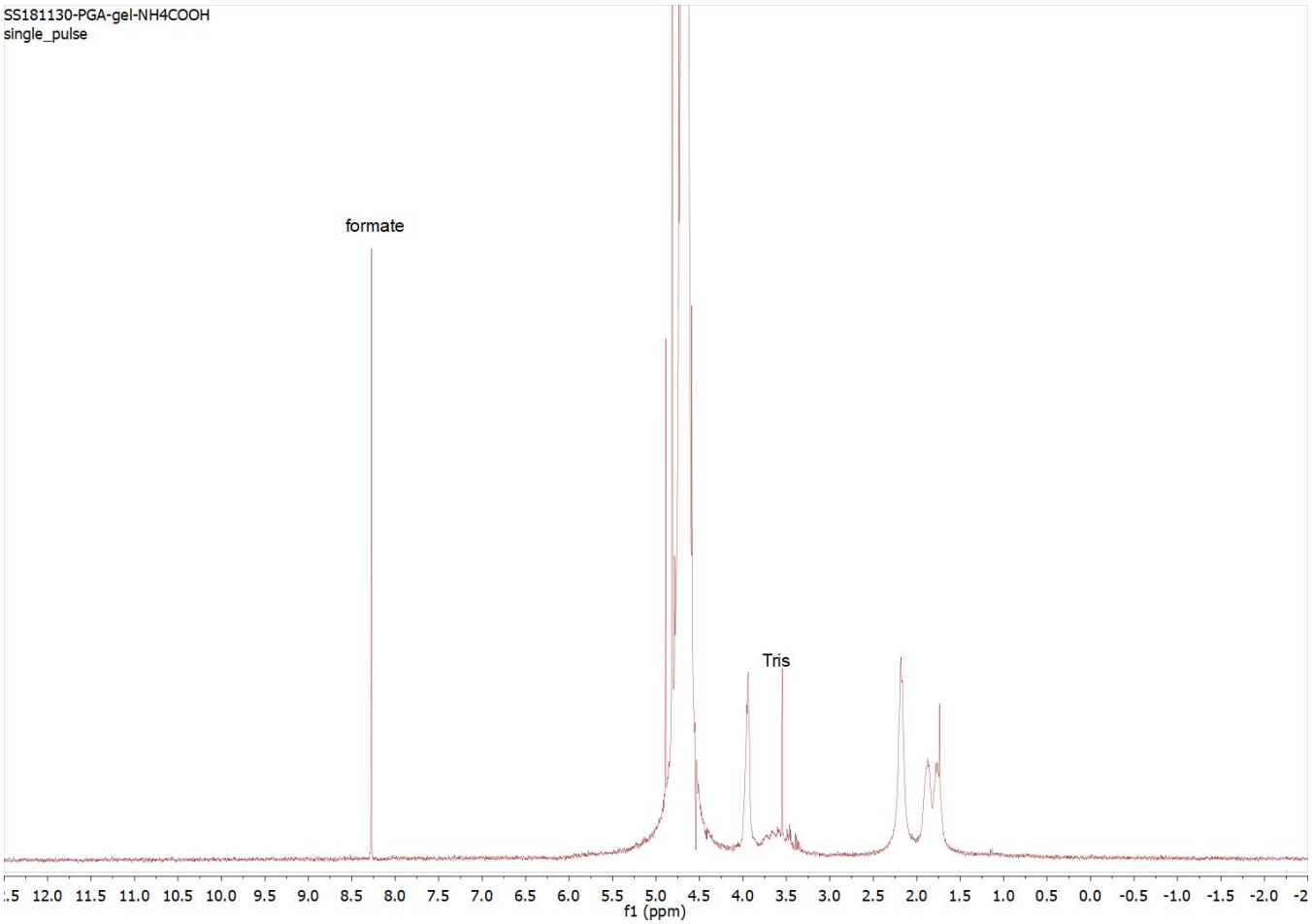

Figure S1. ^{1}H-NMR spectrum (400 MHz, D_2O) of purified PGA.

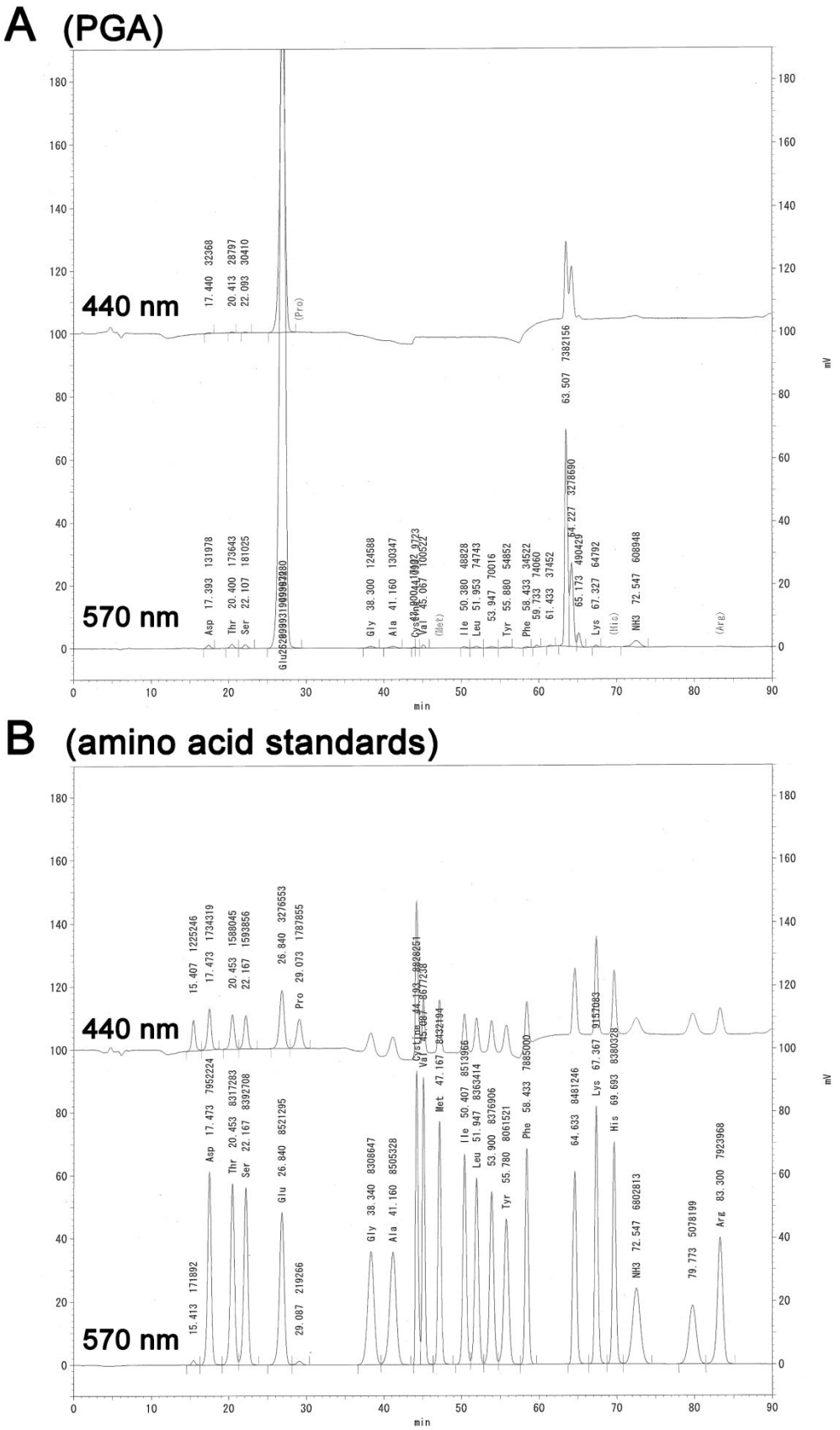

Figure S2. Amino acid composition analysis of purified PGA. (A) PGA and (B) mixture of amino acid standards. Chromatogram was monitored at 440 nm and 570 nm.

Figure S3. Analysis of PGA on 0.8% agarose gel electrophoresis. 1: purified PGA, 2: PGA standard (200–500 kDa), 3: PGA standard (50–100 kDa), 4: PGA standard (15–50 kDa), and 5: PGA standard (3–15 kDa)

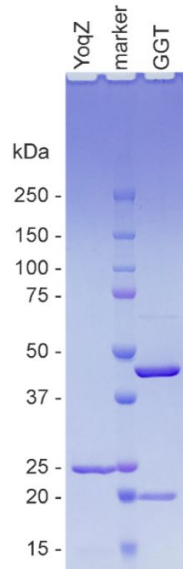

Figure S4. SDS-PAGE analysis of purified YoqZ and GGT.

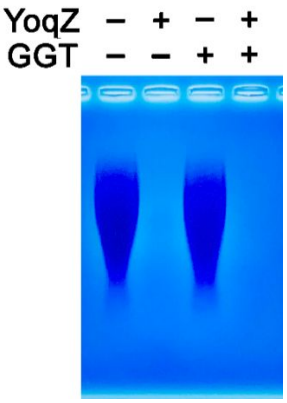

Figure S5. Analysis of enzymatic hydrolysis of PGA on 0.8% agarose gel electrophoresis. Control reactions lacking YoqZ, GGT, or both were also analyzed.

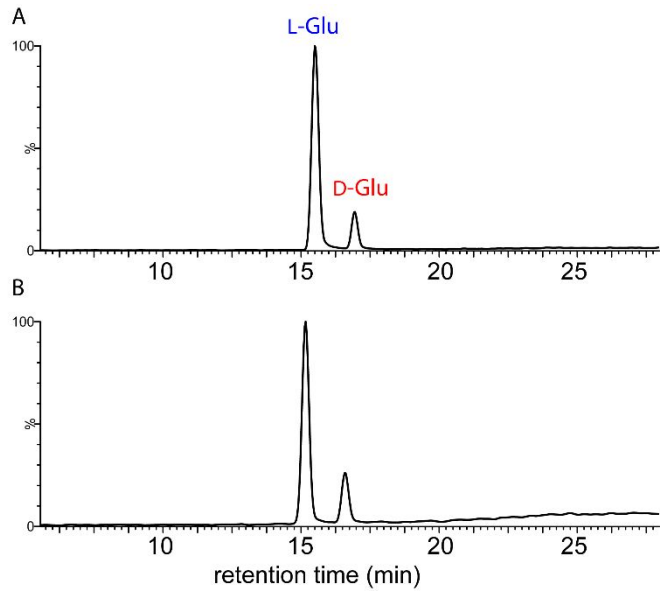

Figure S6. LC-MS analysis of hydrolyzed products. (A) enzymatic hydrolysis and (B) acid hydrolysis. Chromatograms were monitored at *m*/*z* 398.

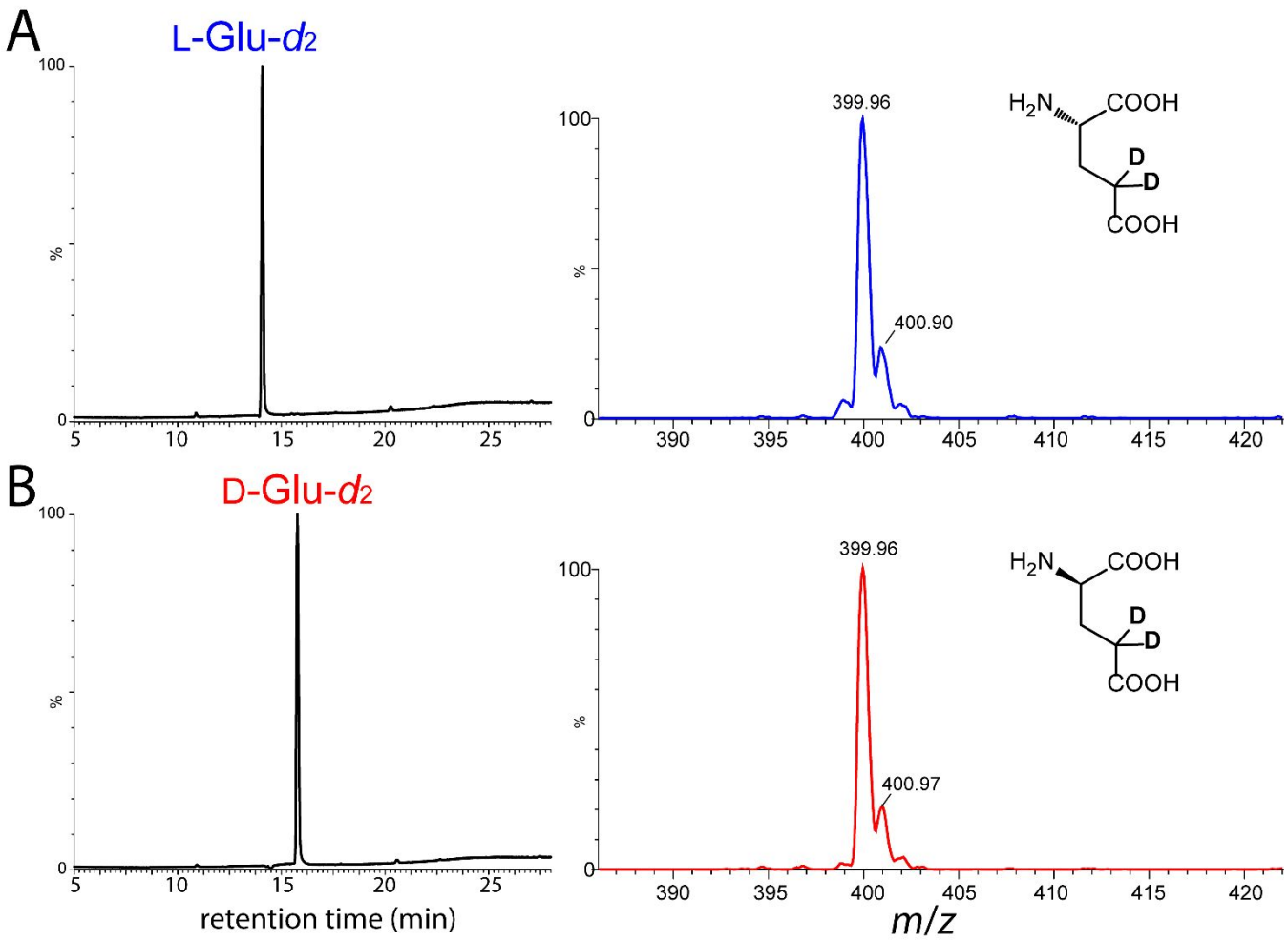

Figure S7. LC-MS chromatogram (left) and spectrum (right) of (A) L-[4,4-$^2H_2$]glutamic acid and (B) D-[4,4-$^2H_2$]glutamic acid. Chromatograms were monitored at *m*/*z* 399±1.5.

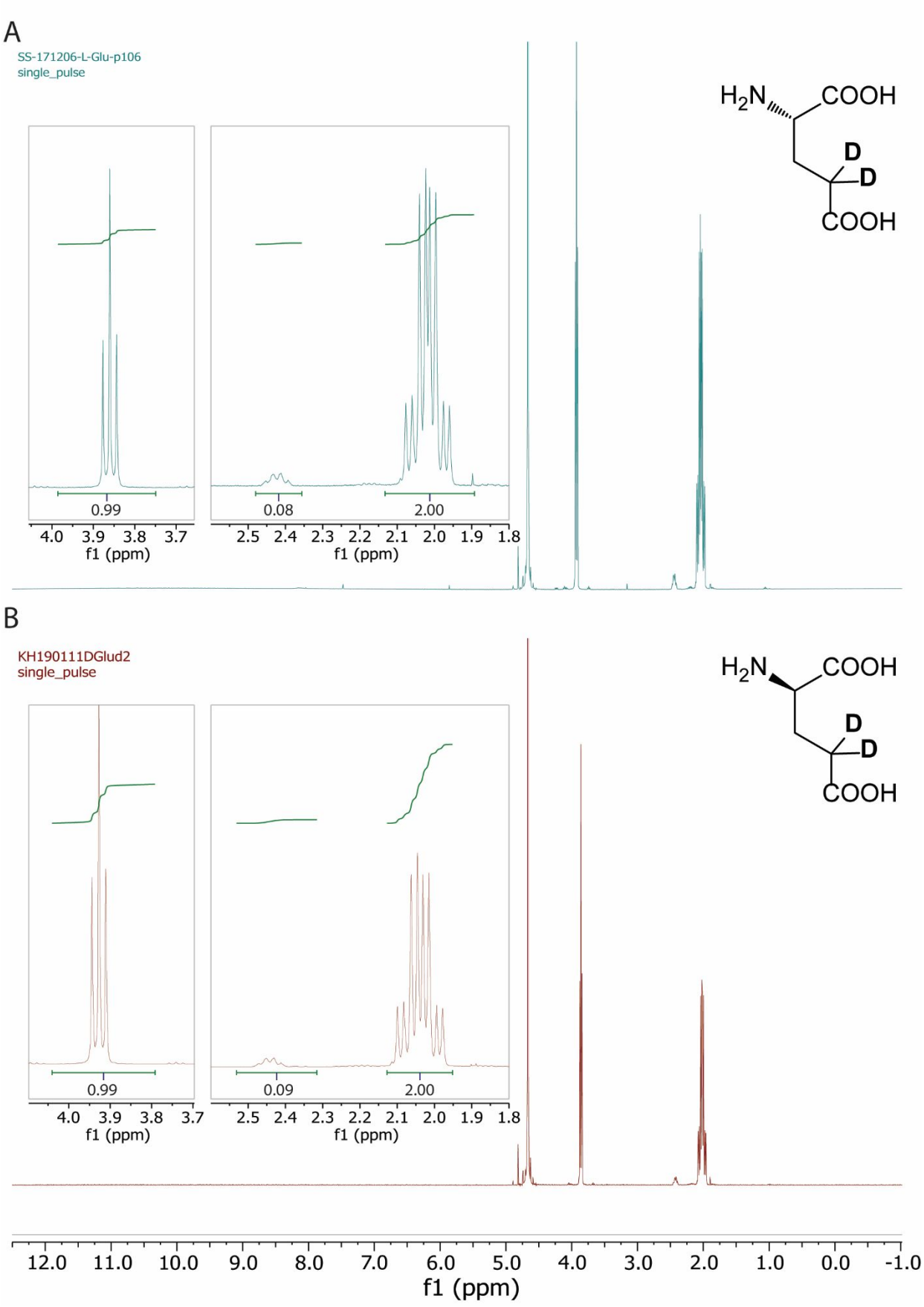

Figure S8. ^{1}H-NMR spectra (400 MHz, D_2O) of (A) L-[4,4-$^{2}H_2$]glutamic acid and (B) D-[4,4-$^{2}H_2$]glutamic acid.

3. References

[1] J. Sambrook and W. Russel, Molecular cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor, NY, 2001.

[2] M. Ashiuchi, K. Soda, and H. Misono, *Biochem*. *Biophys*. *Res*. *Commun*. **263**, *1999*, 6-12.

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[4] K. Wada, M. Irie, H. Suzuki, and K. Fukuyama. *FEBS J.* **277**, *2010*, 1000–1009.

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