

Simultaneous Synthesis and Self-assembly of Cyclic Diphenylalanine at Hydrothermal Condition

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Hydrophobic amino acid, L-phenylalanine, formed insoluble aggregates with unique morphologies at hydrothermal condition. The analyses by infrared spectrum and gel permeation chromatography indicate the highly efficient synthesis of cyclic diphenylalanine probably due to solid-liquid phase separation. The formed cyclic diphenylalanine simultaneously self-assembled to form nanorod structure in the hydrothermal condition.

Recent approach of the hydrothermal organic synthesis for macromolecules and polymers is attracting an interest in the field of green chemistry. Amino acid is one of the low molecules which have been synthesized from carbon dioxide by hydrothermal reaction,¹ and the polymerization of amino acid through dehydration between amino acids has been also reported in sub- or supercritical water.² Cycled hydrothermal treatment of glycine results in the elongation of oligopeptides,³ and rapid quench of temperature increases the yield of oligopeptide, although it is still much less than 10%.⁴

In this study, we report a highly efficient dehydration between hydrophobic amino acids in subcritical water. Amino acid is the monomer composing peptide and protein in vivo, but the self-assembly of the peptide is recently utilized in the field of nanobiotechnology.⁵ We, further, show that the synthesized product forms an unique assembled structure.

L-Phenylalanine (L-Phe) was saturated in distilled water at 25 and 100 °C (170 and 710 mM, respectively), and then the 4.28 mL of L-Phe solutions were loaded into a pressure-resistant vessels (SUS316) with 5 mL of inner volume. The reactor was heated at 220 °C under the pressure of 22 MPa in an electric furnace for various reaction times.

For all the cases, white insoluble aggregates were observed in the reacted solutions, even after they were washed by excess distilled water for the removal of unreacted L-Phe. This implies that the L-Phe was changed to more hydrophobic substances. In order to analyze the chemical structure of the aggregates, attenuated total reflectance Fourier-transform infrared (ATR FT-IR) spectra were measured for these precipitates (Figure 1). For L-Phe, an intense band at 1560 cm⁻¹ corresponding to COO⁻ stretching vibration (Figure 1-(1a)) were observed. For the aggregate formed from a 170 mM L-Phe solution, the IR band disappeared (Figure 1-(1b)), but instead, two weak bands appeared at 1590 and 1660 cm⁻¹, and the band at 1660 cm⁻¹ became strong with both reaction time and L-Phe concentration increased (Figures 1-(1c) and 1-(1d)). The IR band from NH³⁺ group around 2400 cm⁻¹ also decreased by the hydrothermal reaction, and the aggregate formed from the highly-concentrated L-Phe solution showed no absorbance for this band (Figure 1-(2)). Considering that the IR band at 1660 cm⁻¹ can be assigned to νC=O vibration from amide bond, the increase of

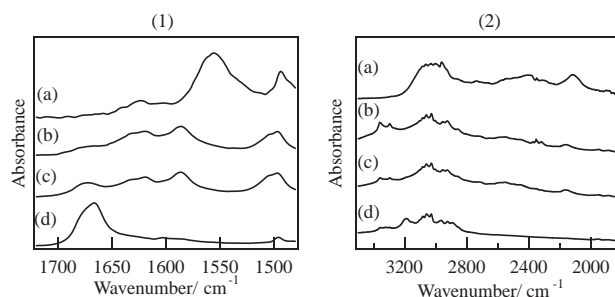


Figure 1. FT-IR spectra of L-Phe (a) and the insoluble aggregates formed at 220 °C in a 170 mM L-Phe solution in 30 min (b) and 120 min (c), in a 710 mM L-Phe solution in 30 min (d).

reaction time and L-Phe concentration leads to the formation of amide bond from carboxyl and amine groups.⁶ The IR bands at 1590 cm⁻¹ is considered to be some COO⁻ vibration, which might indicate residual COO⁻ groups in the insoluble aggregates.

The aggregates formed by the hydrothermal reaction were insoluble in water, but it should be noted that the water-insoluble aggregates could be dissolved in THF; consequently, the dissolved samples were analyzed by gel permeation chromatography (GPC) to identify the molecules composing the aggregates (Figure 2). The chromatogram from the aggregate formed at the low L-Phe concentration (170 mM) in the reaction time of 30 min, had only a single peak (Figure 2a) which was considered to be derived from acetyl phenylalanine by mass analysis. Whereas, the aggregates formed in 120 min showed another component observed at 29 mL of the elution volume where the purchased normal or cyclic diphenylalanines are eluted (Figure 2b). Further, the aggregate formed at the higher concentration (710 mM) mainly showed the dimeric components, independently of the reaction time (Figures 2c and 2d). In order to identify the molecular structure, we measured the molecular weight of the compound at the elution volume of 29 mL by

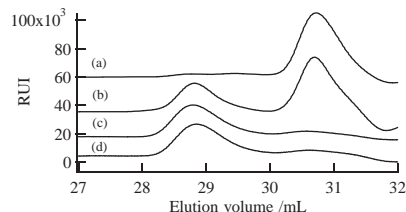


Figure 2. Results of GPC analysis for the synthesized components in insoluble aggregates at concentration of 170 mM at (a) 30 min, (b) 120 min and 710 mM at (c) 30 min, (d) 120 min. A 100 μL of sample (0.2 mg/mL) was applied to a combined GPC column line (Shodex KF801 and 803L) equilibrated with THF. The eluant was monitored by refractive index.

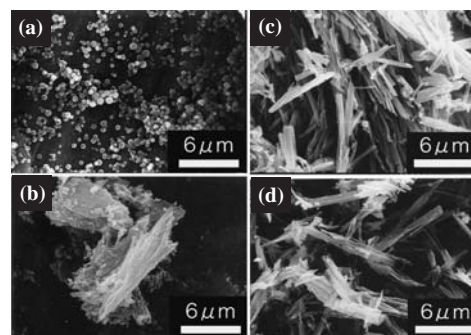
Table 1. The yield of the synthesized precipitates at 220 °C from the 170 and 710 mM L-Phe solutions in the reaction time of 30 and 120 min

Reaction time	170 mM Yield/%	710 mM Yield/%
30 min	0.7	22.2
120 min	6.8	34.0

MALDI-TOF mass spectrum. The spectrum for the dimeric form showed the same molecular mass as the cyclic diphenylalanine (c-Phe-Phe, $[M + H]^+ = 295.064$). The reverse-phase HPLC analysis also supported that most of the components are c-Phe-Phe. These results indicate that the hydrothermal reaction formed c-Phe-Phe rather than normal Phe-Phe. The complete disappearance of the COO^- and NH_3^+ bands in the IR spectrum for the aggregates formed in the highly-concentrated L-Phe solution supports the GPC and mass results (Figure 1d).

Table 1 lists the yield of synthesized c-Phe-Phe by the hydrothermal reaction. The yield was estimated from the results of chromatography. Although longer reaction time led to increase the c-Phe-Phe yield, the L-Phe concentration also promoted the synthesis of c-Phe-Phe. At the high concentration of L-Phe, even at the reaction time of 30 min, the cyclic peptide was obtained with high yield, while the yield of the cyclic peptide formed at the low concentration was as low as 6.8% at 120 min of reaction time. We also changed the reaction temperature and pressure, in the region from 180 to 250 °C, and from 10 to 30 MPa, respectively; however, the change was not critical for the yield of c-Phe-Phe. In the case of glycine, the hydrothermal reaction results in the formation of cyclic diglycine (diketopiperazine). However, the yields are much less than 10%.⁷ This is probably due to the reverse hydrolysis and the subsequent reaction of diketopiperazine with the bi-products. We think that the phase separation for the formation of c-Phe-Phe might be critical for suppressing the further reaction to reduce its yield. Tsukahara et al. reported that the oligomerization of glycine is promoted by the addition of lipid at hydrothermal condition.⁷ This might imply that hydrophobic region by lipid vesicles stabilizes the dehydrated polypeptide at hydrothermal condition. A similar effect can be expected for the aggregates formation in the reaction atmosphere, namely, anti-promoting reaction might proceed by the phase separation of the c-Phe-Phe formed.

In order to observe the morphology of the aggregates formed by the hydrothermal reaction, we took the scanning electron microscopy (SEM) images for the synthesized precipitates (Figure 3). At the low concentration of L-Phe (170 mM), the morphology of the aggregate formed at 30 min was spherical with a diameter of about 500 nm (Figure 3a); however, the shape was changed to rod-like morphology as the reaction time increased (Figure 3b), and at the high L-Phe concentration, only rod-like morphologies were observed, independent of reaction time (Figures 3c and 3d). Previously, Yanagawa et al. reported that amino acids are transformed into some spherical aggregates composed of peptide-like polymers in subcritical water.⁸ They heated the mixture solution of glycine, alanine, valine, and aspartic acids, and observed the spheres with a diameter of 1–3 μm , called microsphere. For the L-Phe concentration, we also found microsphere at 30 min. However, other precipitates were

**Figure 3.** SEM images of the insoluble aggregates after hydrothermal reaction for 30 min (a) and 120 min (b) at the L-Phe concentration of 170 mM, and for 30 min (c) and 120 min (d) at the L-Phe concentration of 710 mM.

rod-like structure (Figure 3). The GPC results clearly demonstrate that the rod structure contains c-Phe-Phe while the dimeric component was not observed for the microsphere. This suggests that the c-Phe-Phe promotes the formation of the rod-like structure. Recently, the formation of nanotube by normal Phe-Phe has been reported. Song et al. revealed that the peptide concentration is critical for the formation of nanotube or spherical vesicles.⁹ Our study is the first report for the formation of microsphere and rod-like structure by c-Phe-Phe. Our results suggest that c-Phe-Phe also behaves like normal Phe-Phe. Reches et al. propose the pathway for the aggregation of Phe-Phe;¹⁰ Phe-Phe, firstly, forms an extended sheet by hydrogen bonds and aromatic stacking interactions, and then the extended sheet is transformed to the spherical or rod structure. The Phe-Phe might form the anisotropic extended sheet to form the formation of rod-structure.

In conclusion, we attained highly efficient synthesis of dehydrated L-Phe, cyclic Phe-Phe, by hydrothermal reaction, probably due to solid–liquid phase separation during the reaction, and also showed that the synthesized cyclic Phe-Phe self-assembles to form rod structure.

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