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Short Communication

Solid-phase peptide synthesis using nanoparticulate amino acids in water

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Abstract: Solid-phase peptide synthesis has many advantages compared with solution peptide synthesis. However, this procedure requires a large amount of organic solvents. Since safe organic solvent waste disposal is an important environmental problem, a technology based on coupling reaction of suspended nanoparticle reactants in water was studied. Fmoc-amino acids are used widely, but most of them show low solubility in water. We prepared well-dispersible Fmoc-amino acid nanoparticles in water by pulverization using a planetary ball mill in the presence of poly(ethylene glycol). Leu-enkephalin amide was prepared successfully using the nanoparticulate Fmoc-amino acid on a poly(ethylene glycol)-grafted Rink amide resin in water.

Keywords: nanoparticles; suspension; solid-phase synthesis; peptide synthesis in water

INTRODUCTION

Solid-phase synthesis has been developed and automated in the field of peptide synthesis [1], and is currently being applied to general organic synthesis along with the spread of combinatorial chemistry [2,3]. However, solid-phase peptide synthesis consumes large quantities of organic solvents. In recent years, the reduced use of organic solvents and utilization of low-toxic reagents are desired from the perspective of green sustainable chemistry [4,5]. For this purpose, we developed an organic-solvent-free, environment-conscious, solid-phase peptide synthesis method using 'water' as an environmentally friendly reaction solvent and reported the synthesis of Leu-enkephalin amide (Tyr-Gly-Gly-Phe-Leu-NH_2) using water-soluble protected amino acids, 2-[phenyl(methyl)sulfonio]ethoxycarbonyl amino acids [6,7], 2-(ethanesulfonyl)ethoxycarbonyl amino acids [8], and 2-(4-sulfophenylsulfonyl)ethoxycarbonyl amino acids [9]. It was the first documented success to carry out the entire process of solid-phase peptide synthesis in water. Since solid-phase synthesis is performed in a two-phase (liquid and solid) medium, an efficient solid-phase reaction in water has not yet been achieved using a sparingly water-soluble protected amino acid. Fmoc-amino acids [10], the most common building blocks for peptide synthesis [11], are sparingly soluble in water, precluding their use for solid-phase synthesis in water. Here, we show a technology using Fmoc-amino acids processed into water-dispersible nanoparticles for solid-phase synthesis in water (Figure 1).

MATERIALS AND METHODS

Preparation of Water-dispersible Fmoc-Amino Acid Nanoparticles

Water-dispersible Fmoc-Phe-OH nanoparticles. An aqueous dispersion of nanoparticulate Fmoc-Phe-OH was prepared by pulverization using a planetary ball mill as follows: A 40 ml agate jar was charged with 1.0 mm diameter precleaned zirconium oxide beads (80 g), Fmoc-Phe-OH (774.8 mg, 2.0 mmol), poly(ethylene glycol) (PEG; mean molecular weight: 4000) (400 mg, 0.1 mmol), and 20 ml of water. The batch was milled at 495 rpm for 2 h. After pulverization, the zirconium oxide beads were removed by filtration with 40 ml of water. The particle size was 265 ± 10 nm.

Water-dispersible Fmoc-Gly-OH nanoparticles. An aqueous dispersion of nanoparticulate Fmoc-Gly-OH was prepared according to the procedure described above. Its formulation consisted of 1.0 mm diameter precleaned zirconium oxide beads (80 g), Fmoc-Gly-OH (891.9 mg, 3.0 mmol), PEG (800 mg, 0.2 mmol), and 20 ml of water. The particle size was 354 ± 26 nm.

Water-dispersible Fmoc-Leu-OH nanoparticles. An aqueous dispersion of nanoparticulate Fmoc-Leu-OH was prepared according to the procedure described above. Its formulation consisted of 1.0 mm diameter precleaned zirconium oxide beads (80 g), Fmoc-Leu-OH (706.8 mg, 2.0 mmol), PEG (1.2 g, 0.3 mmol), and 20 ml of water. The particle size was 265 ± 8 nm.

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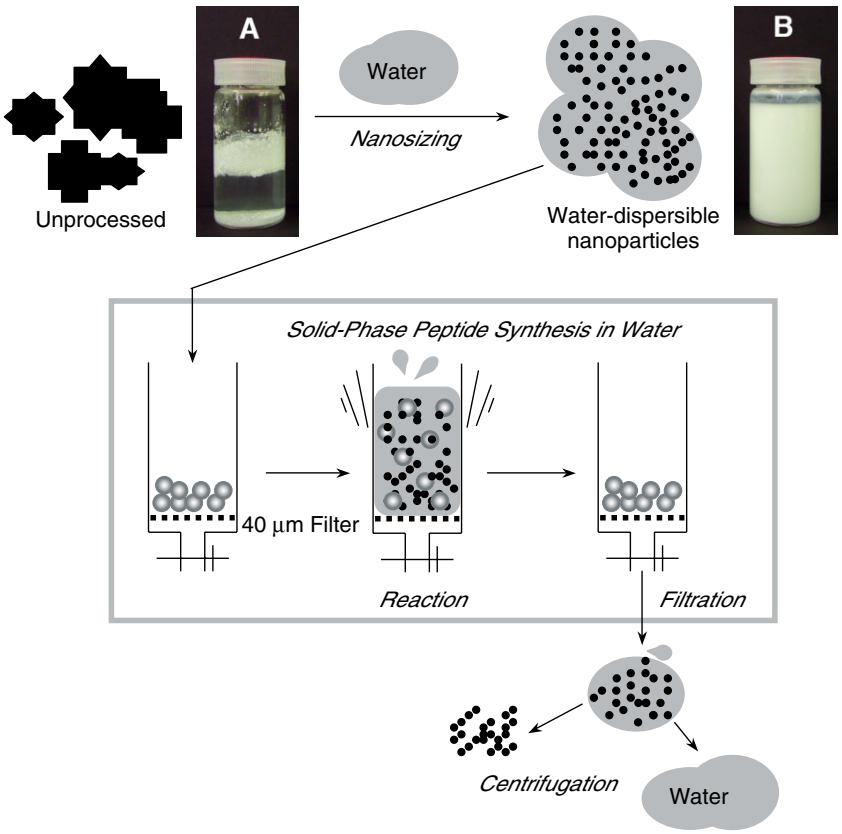

Figure 1 Solid-phase peptide synthesis using nanoparticulate Fmoc-amino acids in water. (A) Unprocessed Fmoc-Phe-OH, (B) Fmoc-Phe-OH aqueous suspension processed into water-dispersible nanoparticles.

Water-dispersible Fmoc-Tyr(tBu)-OH nanoparticles. An aqueous dispersion of nanoparticulate Fmoc-Tyr(*t*Bu)-OH was prepared according to the procedure described above. Its formulation consisted of 1.0 mm diameter precleaned zirconium oxide beads (80 g), Fmoc-Tyr(*t*Bu)-OH (919.2 mg, 2.0 mmol), PEG (1.2 g, 0.3 mmol), and 20 ml of water. The particle size was 440 ± 20 nm.

Coupling Reaction Study of Water-dispersible Fmoc-Phe-OH Nanoparticles onto Leu-PEG-grafted Rink Amide Resin

Leu-PEG-grafted Rink amide resin (48 mg, Leu content, 12.5 μmol) was swelled with water, and then water-dispersible Fmoc-Phe-OH nanoparticles (1.58 ml, 38 μmol) were coupled onto the resin by water-soluble carbodiimide [(WSCD), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride] (7.3 mg, 38 μmol), *N*-hydroxy-5-norbornene-endo-2,3-dicarboximide (HONB) (6.8 mg, 38 μmol), and *N*,*N*-diisopropylethylamine (DIEA) (6.6 μl, 38 μmol). After 15 or 30 min, the resin was washed with water and then the Fmoc group on Phe was removed by treatment with 0.1 N NaOH in 90% EtOH. After drying *in vacuo*, the resin was hydrolyzed with 6 N HCl at 150° for 1.5 h. The Phe and Leu content of the acid hydrolysate was determined and the coupling yield calculated from the Phe to Leu ratio. Coupling rate was 68% after 15 min of reaction and became nearly quantitative after 30 min of reaction.

Solid-phase Synthesis of Leu-enkephalin Amide on PEG-grafted Rink Amide Resin using Water-dispersible Nanoparticles

The solid-phase synthesis was carried out according to the protocol shown in Table 1. PEG-grafted Rink amide resin (185 mg, amino group content, 50 μmol) was swelled with water, and then the water-dispersible nanoparticles of Fmoc-Leu-OH (150 μmol), Fmoc-Phe-OH (150 μmol), Fmoc-Gly-OH (150 μmol), and Fmoc-Tyr(*t*Bu)-OH (150 μmol) were serially coupled to the resin by WSCD (28.6 mg, 150 μmol), HONB (26.8 mg, 150 μmol), and DIEA (26 μl, 150 μmol). Deprotection was carried out with 0.1 N NaOH in 90% EtOH. After completion of the synthetic reaction, the peptide resin (H-Tyr(*t*Bu)-Gly-Gly-Phe-Leu-PEG-grafted Rink amide resin) was washed with EtOH and and dried *in vacuo*. Yield: 205 mg. The resin (205 mg) was treated with TFA–triisopropylsilane–water (92 : 4 : 4, 15 ml) for 2 h at room temperature. The resin was removed by filtration, and then TFA was evaporated to leave an oily material. The residue was dissolved in water, washed with diethylether, and lyophilized. The crude product was purified by preparative HPLC to give an amorphous powder. Purified yield (calculated from the amino group content of the used resin): 22.7 mg, 67%; [α]24 D: +12.5°($c = 1.0$, H_2O), Tof-MS m/z: 555.0 ($[M+1]^+$, $C_{28}H_{39}N_6O_6$ requires 555.64); amino acids analysis: Tyr, 0.94; Gly, 2.00, Phe, 0.96; Leu, 1.03. (average recovery: 92%).

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Table 1 Protocol of in-water, solid-phase synthesis using nanoparticles

Step	Reagents	Time
1	Water	1 min × 5
2	Water-dispersible Fmoc-amino acid nanoparticle, WSCD, HONB, DIEA	1 h
3	Water	1 min × 5
4	Aqueous 50% EtOH	1 min × 2
5	0.1 N NaOH aqueous/90% EtOH	5 min × 3
6	Aqueous 50% EtOH	1 min × 2

In-water Solid-phase Synthesis of Leu-Enkephalin Amide on PEG-grafted Rink Amide Resin using Unprocessed Fmoc-amino Acids

The solid-phase synthesis was carried out according to the protocol shown in Table 1. Since unprocessed Fmoc-amino acids were sparingly soluble in water, the protocol was modified in step 3 in which the washing solvent was changed from water to DMF. PEG-grafted Rink amide resin (555 mg, amino group content, 150 μmol) was swelled with water, and then Fmoc-Leu-OH (450 μmol), Fmoc-Phe-OH (450 μmol), Fmoc-Gly-OH (450 μmol), and Fmoc-Tyr(*t*Bu)-OH (450 μmol) were serially coupled onto the resin by WSCD (85.8 mg, 450 μmol), HONB (80.4 mg, 450 μmol), and DIEA (78 μl, 450 μmol). After completion of the synthetic reaction, the resin was washed with EtOH and dried *in vacuo*. The resin was treated with TFA–triisopropylsilane–water (92 : 4:4, 40 ml) for 2 h at room temperature. The resin was removed by filtration and TFA was evaporated to leave an oily material. The residue was dissolved in water, washed with diethylether, and lyophilized to give an amorphous material. Yield: trace. The crude product was analyzed by HPLC.

In-water Solid-phase Synthesis of Leu-Enkephalin Amide on PEG-grafted Rink Amide Resin using Unprocessed Fmoc-amino Acids with PEG

The solid-phase synthesis was carried out according to the protocol shown in Table 1. Since unprocessed Fmoc-amino acids were sparingly soluble in water, the protocol was modified in step 3, in which the washing solvent was changed from water to DMF. PEG-grafted Rink amide resin (555 mg, amino group content, 150 μmol) was swelled with water, and then Fmoc-Leu-OH (450 μmol), Fmoc-Phe-OH (450 μmol), Fmoc-Gly-OH (450 μmol), and Fmoc-Tyr(*t*Bu)-OH (450 μmol) were serially coupled onto the resin by WSCD (85.8 mg, 450 μmol), HONB (80.4 mg, 450 μmol), DIEA (78 μl, 450 μmol) and PEG (200 mg). After completion of the synthetic reaction, the resin was washed with EtOH and dried *in vacuo*. The resin was treated with TFA–triisopropylsilane–water (92 : 4 : 4, 40 ml) for 2 h at room temperature. The resin was removed by filtration and then TFA was evaporated to leave an oily material. The residue was dissolved in water, washed with diethylether, and lyophilized to give an amorphous material. Yield: trace. The crude product was analyzed by HPLC.

RESULTS AND DISCUSSION

Nanosizing of particles is a part of particle design to improve the function of sparingly soluble drugs [12,13]. Grinding particles to nanosize can lead to increased specific surface area and homogeneous mixing of multiple components. We therefore predicted that conversion of Fmoc-amino acids into homogeneously water-dispersible nanoparticles would enable increased specific surface area and homogeneous mixing with the resin in water, and resulting in an unproblematic progress of the solid-phase reaction in water according to the Fmoc approach. There have been no available reports of reactions in water using such water-dispersible nanoparticles as building blocks, as far as we are aware. When synthesis in water is carried out using protected water-soluble amino acids, the excess amount of protected water-soluble amino acids are dissolved in water after the reaction, causing possible contamination of the water. However, water-dispersible nanoparticles can be easily removed from the reaction by filtration and water-washing of the resin. Also, the dispersible nanoparticles in the filtrate can also be easily separated from water by deposition or sedimentation through ultracentrifugation or other methods. The present nanoparticle-based technology will represent a major step in the development of environmentally friendly solid-phase chemical synthesis methods.

First, we selected Fmoc-Phe-OH, which has an aromatic ring in the side chain and is highly hydrophobic, to convert into water-dispersible nanoparticles. A dispersion additive, PEG [14,] was added to Fmoc-Phe-OH and the mixture was ground for 2 h at room temperature in a planetary ball mill containing water with zirconia beads. The formulation consisted of 2.0 mmol of Fmoc-Phe-OH, 400 mg of PEG, 20 ml of water, and 80 g of zirconia beads. The size of the resulting water-dispersible nanoparticles (Figure 1(B)) was determined by dynamic light scattering analysis (DLS) to be 265 ± 10 nm. A scanning electron microscope (SEM) image also revealed nanosized particles (Figure 2(B)). Then, we established the conditions for in-water, solid-phase coupling reaction using the water-dispersible nanoparticles. As the solid phase the Leu-PEG-grafted Rink amide resin [15,16] that was swollen in water was selected, and three equivalents of Fmoc-Phe-OH nanoparticles was used to the content of Leu in the resin. WSCD [17] was used as the coupling reagent and HONB [18] and DIEA were used as additives. After the reaction, nanoparticles were removed from the reaction mixture by water-washing and filtration. The ninhydrin Kaiser test [19] of the resin was negative after a 30-min reaction, indicating effortless progress of coupling. Coupling ratio of Phe onto the resin was calculated from amino acid analysis data of the resin after 15- and 30-min reactions. The coupling rate was 68% after 15 min of reaction and became nearly quantitative

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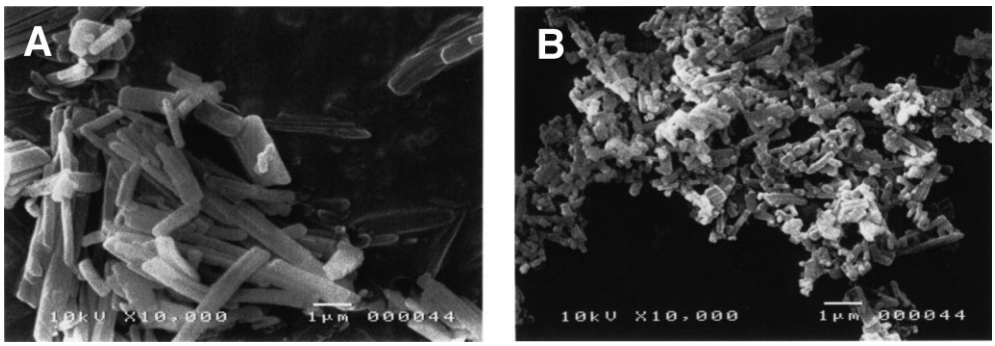

Figure 2 (A) An SEM image of unprocessed Fmoc-Phe-OH, (B) An SEM image of water-dispersible nanoparticulate Fmoc-Phe-OH.

after 30 min. These findings indicate that nanoparticle-based coupling reaction was completed in a short time (30 min) even though the reaction was accomplished on solid phase in water. This rapid completion of reaction is attributed mainly to the homogeneous mixing of nanoparticles with the resin. The above reaction was more effortless than expected, given that at least 1 h was necessary when four equivalents of amino acids was used in previously reported in-water [8], solid-phase coupling reactions using water-soluble protected amino acids. The promoted reaction may be induced by a new physical property that was acquired from nanosizing.

We selected a five-residue opioid peptide, Leu-enkephalin amide, as a model peptide to perform in-water, solid-phase reactions using Fmoc-amino acids processed into water-dispersible nanoparticles. PEG-grafted Rink amide resin was used as the solid support. WSCD was used as a coupling reagent. HONB and DIEA were used as additives. Deprotection of Fmoc group was carried out with 0.1 N NaOH in 90% EtOH since the Fmoc-derived fluorine compound was sparingly soluble in water. In accordance with the protocol shown in Table 1, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, and Fmoc-Tyr(*t*Bu)-OH, each in a form of water-dispersible nanoparticles, were serially coupled onto the resin and a Fmoc-Tyr(*t*Bu)-Gly-Gly-Phe-Leu-PEG-grafted Rink amide resin was obtained. After removal of the Fmoc group, cleavage of the partially protected peptide from the resin and final deprotection were carried out with TFA. Results of the HPLC analysis for Leu-enkephalin amide obtained by solid-phase synthesis in water are shown in Figure 3(C). Leu-enkephalin amide provided the main peak. The overall yield calculated from the amino group content of the used resin was 67%.

We also prepared Leu-enkephalin amide by solid-phase synthesis using unprocessed Fmoc-amino acids in water and in an aqueous PEG solution. HPLC analysis results of the crudes after cleavage from the resin, as shown in Figure 3(A) and (B), reveal no major peak at the retention time for Leu-enkephalin amide. The in-water synthesis using unprocessed Fmoc-amino acids was not achieved, even in the presence of the dispersible agent PEG.

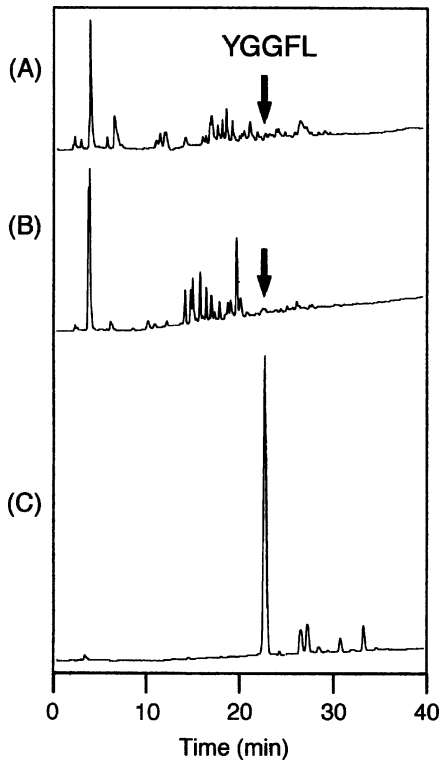

Figure 3 HPLC analysis data after cleavage from the resin of Leu-enkephalin amide prepared by in-water, solid-phase synthesis using (A) unprocessed Fmoc-amino acids, (B) unprocessed Fmoc-amino acids with PEG, and (C) water-dispersible nanoparticulate Fmoc-amino acids. Column, DAISOPAK SP-120-5-ODS-B (2.5 × 250 mm). Flow rate, 1 ml/min. Eluent, CH_3CN/H_2O containing 0.05% TFA. Gradient: 10/90–50/50 (40 min).

In the present study, we developed a new peptide synthesis method based on nanosized building blocks. There have been no reports of in-water or solid-phase reactions using dispersible nanoparticles as building blocks. While traditional solid-phase methods are based on 'reaction between a solid reactant and a reactant dissolved in an organic solvent', the present method is based on 'reaction between a solid reactant

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and an aqueous nanoparticulate suspension' and has promising applications in the field of nanochemistry, where water-insoluble materials are suspended in water as nanoparticulate reactants. Green sustainable chemistry is regarded as a challenge in science and technology to achieve reduced use of organic solvents and utilization of low-toxic reagents. However, most solid-phase syntheses are currently carried out in organic solvents, and few efforts have been made to carry out reactions in water. Low water solubility of reagents and building blocks presents one of the obstacles in the effort to carry out reactions in water. The present study proposes a solution to address the low solubility of building blocks and offers a new solid-phase synthesis method using the 'environmentally friendly solvent', water. Reaction characteristics for nanosized building blocks will be explored to trigger a new methodology for synthetic reactions.

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