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Peptide synthesis 'in water' by a solution-phase method using water-dispersible nanoparticle Boc-amino acid

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Regulatory pressure has compelled the chemical manufacturing industry to reduce the use of organic solvents in synthetic chemistry, and there is currently a strong focus on replacing these solvents with water. Here, we describe an efficient in-water solution-phase peptide synthesis method using Boc-amino acids. It is based on a coupling reaction utilizing suspended water-dispersible nanoparticle reactants. Using this method, peptides were obtained in good yield and with high purity. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: nanoparticles; suspension; solution-phase synthesis; peptide synthesis in water; Boc strategy; green chemistry

Introduction

Since the early 20th century, the use of organic solvents in chemical reactions has led to a great leap forward in modern organic chemistry. More stringent rules regulating the use and disposal of organic solvents are increasingly restricting chemical synthesis alternatives, and there is an urgent need to develop non-hazardous alternatives in order to achieve green sustainable chemistry [1,2]. Owing to the vast importance of peptides in biological processes, there is an escalating need for synthetic peptides to be used in a wide variety of applications. However, the consumption of organic solvent is extremely large in chemical peptide syntheses because of the multiple condensation steps in organic solvents. The problem with organic solvents is not so much their use but the seemingly inherent inefficiencies associated with the disposal of waste. There is a compelling need for new peptide synthesis technologies that do not damage the environment. From the viewpoint of green sustainable chemistry, the best solvent is no solvent. If a solvent is needed, then water has much to offer: It is non-toxic, non-inflammable, abundantly available and inexpensive [3]. Seeking a more environmentally balanced method of peptide synthesis, we focused on developing an organic solvent-free synthetic method using water, an environmentally friendly solvent.

Consumption of organic solvents can be radically reduced by developing alternative methods that utilize water. In conventional organic syntheses, reactant molecules are routinely dissolved in solvents to carry out the reaction efficiently. Peptide synthesis can be carried out in water via chemical conversion of protected amino acids to water-soluble forms [4–7]. This procedure, however, requires an additional conversion step in the synthetic process, and this is undesirable in terms of preparation cost, resource saving and energy conservation. Thus, development of new techniques other than chemical conversion is a challenging issue. In current peptide synthesis methods, the most common building blocks are the *t*-butyloxycarbonyl (Boc)- and 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids [8,9], which are highly soluble in organic solvents. These molecules are sparingly soluble in water and are considered inappropriate for in-water synthesis. We recently reported a new technique, solid-phase peptide synthesis in water, which utilizes Fmoc-amino acids converted to water-dispersible nanoparticles [10,11].

In industrial syntheses, peptide products such as Aspartame (Asp-Phe-OMe), Leuplin® (an LHRH analog used for cancer therapy) (Takeda Pharmaceutical Co. Ltd., Osaka, Japan) and so on are produced primarily by solution-phase peptide synthesis. The Boc strategy is well known to be suitable for industrial chemistry and green chemistry, because only gases are generated, without any other by-products produced by deprotection of the Boc group. Gases require less disposal energy than do solid wastes generated by deprotection of other protecting groups such as the Fmoc group. Here, we describe an in-water solution-phase peptide synthesis method in which Boc-amino acids are converted to water-dispersible nanoparticles.

In recent years, utilization of nanoparticle-based technology has emerged as a strategy to tackle formulation problems associated with poorly water-soluble drugs [12–14]. Accordingly, reduction of drug particle sizes to the nano-scale provides an increased surface area and allows homogenous mixing of multiple components, leading to improved *in vivo* drug performance. When Boc-amino

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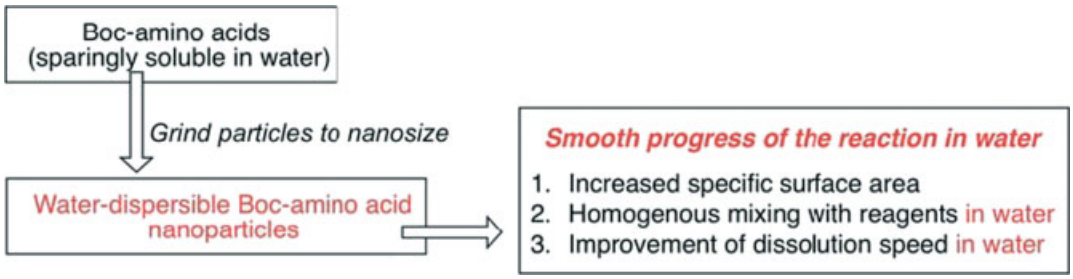

Figure 1. New physical properties and reactivities acquired by water-dispersible nanoparticulation.

acids are converted to nanoparticles homogeneously dispersed in water, the specific surface area is increased and water dissolution speed is improved. Even when water-insoluble Boc-amino acids are used, the reaction can be dramatically accelerated. This suggests that synthesis of peptides in water according to the Boc strategy is a promising direction to pursue. (Figure 1)

Materials and Methods

Boc-amino acids were purchased from Watanabe Chemical Industries, Ltd. A planetary ball mill, model pulverisette 7 (Fritsch GmbH, Markt Einersheim, German), was used for pulverization to prepare water-dispersible nanoparticles. The size of particles was determined by a dynamic light scattering (DLS) analysis, using instrument model LB-500 (Horiba Instruments Inc.). A nanoparticle image was taken by a scanning electron microscope (SEM), model JSM-5300LV (JEOL Ltd., Tokyo, Japan). Amino acid ratios in an acid hydrolysate were determined with a Waters Pico Tag amino acid analyzer. Reversed phase HPLC was performed using Waters model 600 equipment with a DISOPAK column and a gradient system consisting of acetonitrile/water containing 0.05% TFA. Optical rotations were determined with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co., Tokyo, Japan). Mass spectra were measured with a Kratos MALDI IV mass spectrometer (Simadzu Co., Kyoto, Japan) using the TOF technique.

General Procedure for Preparation of Water-Dispersible Nanoparticle Boc-Amino Acids

An aqueous dispersion of nanoparticulate Boc-amino acids was prepared by pulverization using a planetary ball mill as follows: A 40-ml agate jar was charged with 1.0-mm diameter pre-cleaned zirconium oxide beads (80 g), Boc-Phe-OH (530.6 mg, 2.0 mmol), PEG (average molecular weight 4000 g/mol, 400 mg, 0.1 mmol) and 20 ml of water. The batch was rolled at 495 rpm for 4 h. After pulverization, the zirconium oxide beads were removed by filtration with 40 ml of water. The particle sizes were determined by DLS analysis and the particles had the following characteristics:

Boc-Phe-OH nanoparticles: particle size = 578 ± 48 nm
Boc-Gly-OH nanoparticles: particle size = 712 ± 36 nm
Boc-Tyr(*t*Bu)-OH nanoparticles: particle size = 687 ± 32 nm.

An aqueous dispersion of nanoparticulate Boc-amino acids having benzyl (Bzl) type side chain protection groups was prepared by pulverization using a planetary ball mill as follows: A 40-ml agate jar was charged with 0.5-mm diameter pre-cleaned zirconium oxide beads (80 g), Boc-Tyr(Bzl)-OH (494.3 mg, 1.0 mmol) and 20 ml of aqueous 0.4% Triton X-100 solution. The batch was rolled at 495 rpm for 4 h. After pulverization, the zirconium oxide beads were removed by filtration with 60 ml of aqueous 0.4% Triton X-100 solution. The particle sizes were determined by DLS analysis and they had the following characteristics:

Boc-Tyr(BrZ)-OH nanoparticles: particle size = 435 ± 118 nm

In-Water Coupling Reaction of Water-Dispersible Boc-Phe-OH Nanoparticle with H-Leu-NH_2 using Water-Soluble Coupling Reagents

H-Leu-NH_2·HCl (166 mg, 1.0 mmol) was dissolved in 10 ml of water, and then water-dispersible Boc-Phe-OH nanoparticles (60 ml, 2.0 mmol) were added. Several coupling methods using WSCI [1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride] (382 mg, 2.0 mmol) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) (552 mg, 2.0 mmol) were examined. *N*-hydroxy-5-norbornene-*endo*-2,3-dicarboximide (HONB) (450 mg, 2.0 mmol), *N*-hydroxysuccinimide (HOSu) (230 mg, 2.0 mmol) or 3-sulfo-*N*-hydroxysuccinimide (sulfo-HOSu) (436 mg, 2.0 mmol) was used as a coupling additive. *N*,*N*-DIEA (348 µl, 2.0 mmol) was used in the WSCI methods, and *N*MM (192 µl, 2.0 mmol) was used in the DMTMM method. After stirring overnight, the reaction mixture was filtered to collect the precipitates. The precipitates, which contained the crude peptide, were directly applied to analytical HPLC, and the yields and purities were measured.

General Procedure for Synthesis of Peptides using Water-Dispersible Boc-Amino Acid Nanoparticles, with Boc-Gly-Phe-Leu-NH_2 as an Example

Boc-Phe-Leu-NH_2 (674 mg, 1.0 mmol) was treated with 20 ml of TFA) for 1 h at room temperature. The TFA solution was concentrated to a residue *in vacuo*, to which 1 ml of solution 4.0 mol/l HCl in dioxane was added. The residue was triturated with diethyl ether. The precipitate was collected by filtration and dried over sodium hydroxide pellets *in vacuo*. H-Phe-Leu-NH_2·HCl was thus obtained and dissolved in 20 ml of water. Water-dispersible Boc-Gly-OH nanoparticles (60 ml, 2.0 mmol) were mixed and then DMTMM (552 mg, 2.0 mmol) and NMM (196 µl, 2.0 mmol) were added. After stirring at room temperature overnight, the precipitate was collected by filtration and washed with water. The residue was dried *in vacuo* to obtain crude peptide, which was directly applied to analytical HPLC, and the yields and purities were measured. The yields and characteristics were Boc-Gly-Phe-Leu-NH_2: Yield 82% (calculated from analytical HPLC), HPLC analytical purity 90%. Crude peptide was crystallized from ethyl acetate and diethyl ether. mp 155–157 °C; $[\alpha]_D^{24}$ + 55.7 (*c* 1.0 in MeOH); *m/z* (MALDI-MS) 457.54 ($[M+Na]^+$, $C_{22}H_{34}N_4NaO_5$ calculated *m/z* = 457.52); Boc-Phe-Leu-NH_2: white material; mp

Journal of PeptideScience

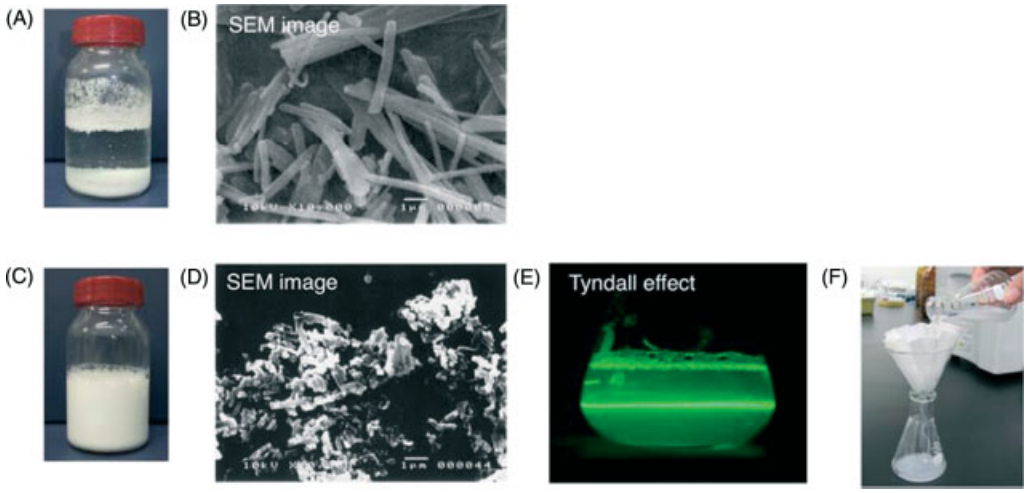

Figure 2. (A) Photo image of unprocessed Boc-Phe-OH. (B) SEM image of unprocessed Boc-Phe-OH. (C) Photo image of water-dispersible nanoparticle Boc-Phe-OH. (D) SEM image of water-dispersible nanoparticle Boc-Phe-OH. (E) Water-dispersible Boc-Phe-OH nanoparticles showed the Tyndall phenomenon upon laser irradiation. (F) Water-dispersible nanoparticle Boc-Phe-OH readily passed through a filter paper.

176–178 °C; $[\alpha]_D^{24}$ −20.2 (*c* 1.0 in MeOH); *m/z* (MALDI-MS) 400.86 ($[M+Na]^+$, $C_{20}H_{31}N_3NaO_4$ calculated $m/z = 400.47$); Boc-Gly-Gly-Phe-Leu-NH_2: white material, mp 137–140 °C; $[\alpha]_D^{24}$ +64.4 (*c* 1.0 in MeOH); *m/z* (MALDI-MS) 514.27 ($[M+Na]^+$, $C_{24}H_{37}N_5NaO_6$ calculated $m/z = 514.57$); Boc-Tyr(*t*Bu)-Gly-Gly-Phe-Leu-NH_2: yellowish material; $[\alpha]_D^{24}$ +68.6 (*c* 1.0 in MeOH); *m/z* (MALDI-MS) 733.27 ($[M+Na]^+$, $C_{37}H_{54}N_6NaO_8$ calculated $m/z = 733.85$).

Preparation of Leu-Enkephalinamide (H-Tyr-Gly-Gly-Phe-Leu-NH_2·TFA)

The protected pentapeptide Boc-Tyr(*t*Bu)-Gly-Gly-Phe-Leu-NH_2 (710 mg, 1.0 ml) was treated with 20 ml of TFA for 1 h at room temperature. The TFA solution was concentrated to a residue *in vacuo*. The residue was purified by preparative HPLC to give an amorphous powder. The yield was 51%; $[\alpha]_D^{24}$ + 12.6 (*c* 1.0 in H_2O); *m/z* (MALDI-MS) 555.31 ($[M+H]^+$. $C_{28}H_{39}N_6O_6$ calculated $m/z = 555.64$); amino acid analysis: Tyr, 0.94; Gly; 2.03, Phe, 0.92; Leu, 1.01 (average recovery: 92%).

Results and Discussion

Nanoparticles can be produced by dispersion-based processes. Wet milling is an attrition-based process in which the insoluble material is dispersed in the aqueous-based surfactant solution, and the resulting material is converted into water-dispersible nanoparticles. First, we selected Boc-Phe-OH, which has a hydrophobic lipophilic aromatic ring in its side chain, to convert to water-dispersible nanoparticles. In the presence of PEG [15] as a dispersant, Boc-Phe-OH was ground with zirconia beads in water using a planetary ball mill for 2 h at room temperature. The beads were then removed by filtration and water-dispersible nanoparticles of Boc-Phe-OH were obtained. SEM images of the nanoparticles are shown in Figure 2: Panels (b) and (d) show the nanoparticles before and after grinding, respectively. Nano- or submicron-sized particles were observed. The nanoparticles dispersed in water were found by DLS to have a mean diameter of 578 ± 48 nm. The obtained nanoparticles showed the Tyndall phenomenon upon laser irradiation and readily passed through a filter paper (ADVANTEC Filter Paper No.2). In the Boc strategy, the hydrophobic Bzl type groups are generally the first choice for the side chain protection step. We also successfully prepared water-dispersible Boc-Tyr(BrZ)-OH, which has a hydrophobic BrZ side chain protecting group, as a nanoparticle formulation using the same wet milling process in the presence of another dispersant, an aqueous 0.4% Triton X-100 solution. The Boc-Tyr(BrZ)-OH nanoparticles dispersed in water were found by DLS to have a mean diameter of 435 ± 118 nm.

Table 1. In-water coupling reaction using water-dispersible nanoparticles[a]

Entry	Reagent	Additive	Base	Yield (%)[b]
1	DMTMM	–	NMM	89
2	WSCI	HONB	DIEA	25
3	WSCI	HOSu	DIEA	32
4	WSCI	Sulfo-HOSu	DIEA	71

[a] Reactions were carried out between H-Leu-NH_2 and water-dispersible Boc-Phe-OH nanoparticles.
[b] The yield of peptides was calculated from analytical HPLC profiles.

To evaluate the feasibility of using the water-dispersible Boc-amino acid nanoparticles as building blocks for solution-phase in-water peptide synthesis, we examined in-water coupling reactions between water-dispersible Boc-Phe-OH nanoparticles and Leu-NH_2. The reaction was carried out by several coupling methods using the water-soluble coupling reagents WSCI [16] and DMTMM [17,18]. As shown in Table 1, the coupling was more favorable with DMTMM than with the WSCI combination containing the additives HONB [19], HOSu [20,21] or sulfo-HOSu [22]. The in-water coupling reaction using DMTMM in the presence of NMM resulted in good yields (89%).

Next, solution-phase in-water synthesis of Leu-enkephalinamide was carried out using water-dispersible nanoparticles of Boc-amino acids prepared as described above. Figure 3 shows the synthetic scheme. DMTMM was used as the coupling reagent. The peptide was deprotected with TFA and the TFA salt was exchanged for the HCl salt using a 4.0 mol/l HCl-dioxane solution. Coupling was carried out in water by mixing water-dispersible nanoparticles of

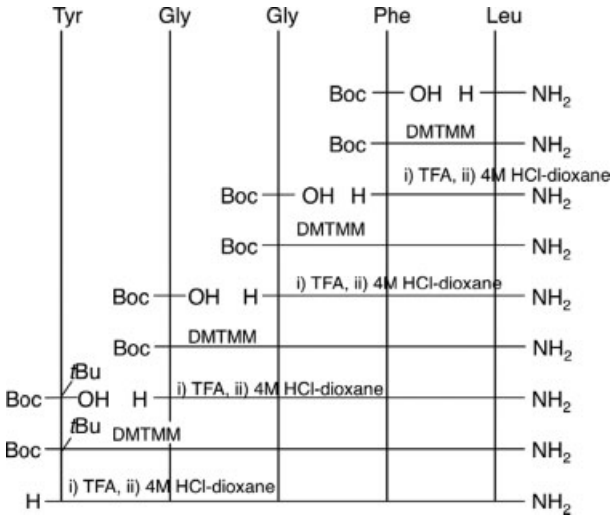

Figure 3. Synthetic scheme of Leu-enkephalinamide.

a Boc-amino acid with an amino component, adding DMTMM and then stirring the mixture under neutral conditions for 12 h at room temperature (Figure 4). As nanoparticles readily pass through a micro-pore filter and the reagent is water-soluble, post-coupling treatment consists only of removing the unreacted nanoparticles and reagent-mediated by-products by filtration to obtain the main product. Figure 5 shows HPLC profiles of the crude main products obtained after filtration. Each protected peptide fragment showed a single predominant peak, even without special purification steps other than filtration, showing that these peptides were obtained with high purity. Table 2 shows the yield and purity of the crude product of each coupling reaction. In all reactions, peptides were obtained with a yield of 82% or higher and with a purity of 90% or higher, showing the feasibility of efficient in-water coupling reactions using water-dispersible nanoparticles. After the final deprotection step, the target product Leu-enkephalinamide was obtained with a total synthetic yield of 51%. In this study, all the amino components were in a water-soluble salt form. If the sparingly soluble amino component was used, a purification step would have been needed that would include not only filtration but also some other purification technique(s), e.g. an extraction or chromatography method.

Table 2. Yield and purity of in-water synthetic peptides[a]

Synthetic peptide	Yield (%)[b]	Purity (%)[b]
Boc-Phe-Leu-NH_2	89	90
Boc-Gly-Phe-Leu-NH_2	82	90
Boc-Gly-Gly-Phe-Leu-NH_2	84	92
Boc-Tyr(*t*Bu)-Gly-Gly-Phe-Leu-NH_2	86	93

[a] In-water coupling reactions were carried out with DMTMM. Purity of peptides was calculated from analytical HPLC profiles.
[b] Yield and purity of peptides was calculated from analytical HPLC profiles.

In this study, water-dispersible nanoparticles of Boc-amino acids were prepared in a planetary ball mill, and Leu-enkephalinamide was successfully synthesized in water by Boc chemistry using these nanoparticles. In addition, coupling reactions mediated by DMTMM in water containing water-dispersible nanoparticles were found to progress smoothly, resulting in high yield and high purity products. Hydrophobic molecules tend to associate in water. Providing a nano-scale reaction field that promotes molecular associations might improve the efficiency of these types of reactions. In the case of water-dispersible nanoparticles with poor solubility in water, it is likely that the nanoparticles are homogeneously dispersed in water and recognize each other by their hydrophobicity. Therefore, it should be possible to establish a nano-scale, hydrophobic reaction field in aqueous media to produce high yields of product. Nanoparticles have novel, interesting physical properties, like much smaller diameters and much larger specific surface areas, compared to common particles.

In general, most organic compounds produced as intermediates in industrial syntheses are sparingly soluble in water and are thus not suitable for in-water reactions. However, if a technology enabling efficient in-water reactions using compounds with poor water solubility, such as the method described here, is established, then it could replace standard organic synthesis methods with more environmentally friendly water-using processes.

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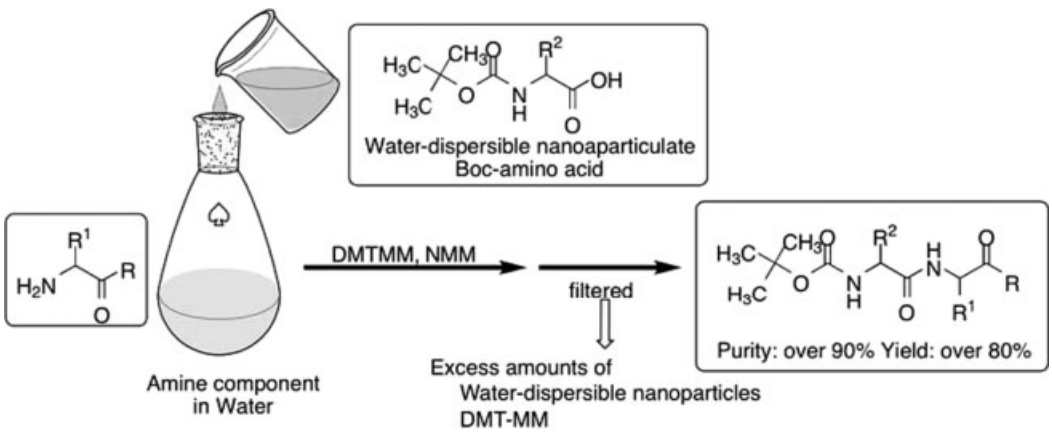

Figure 4. In-water coupling reaction using a water-dispersible nanoparticle Boc-amino acid.

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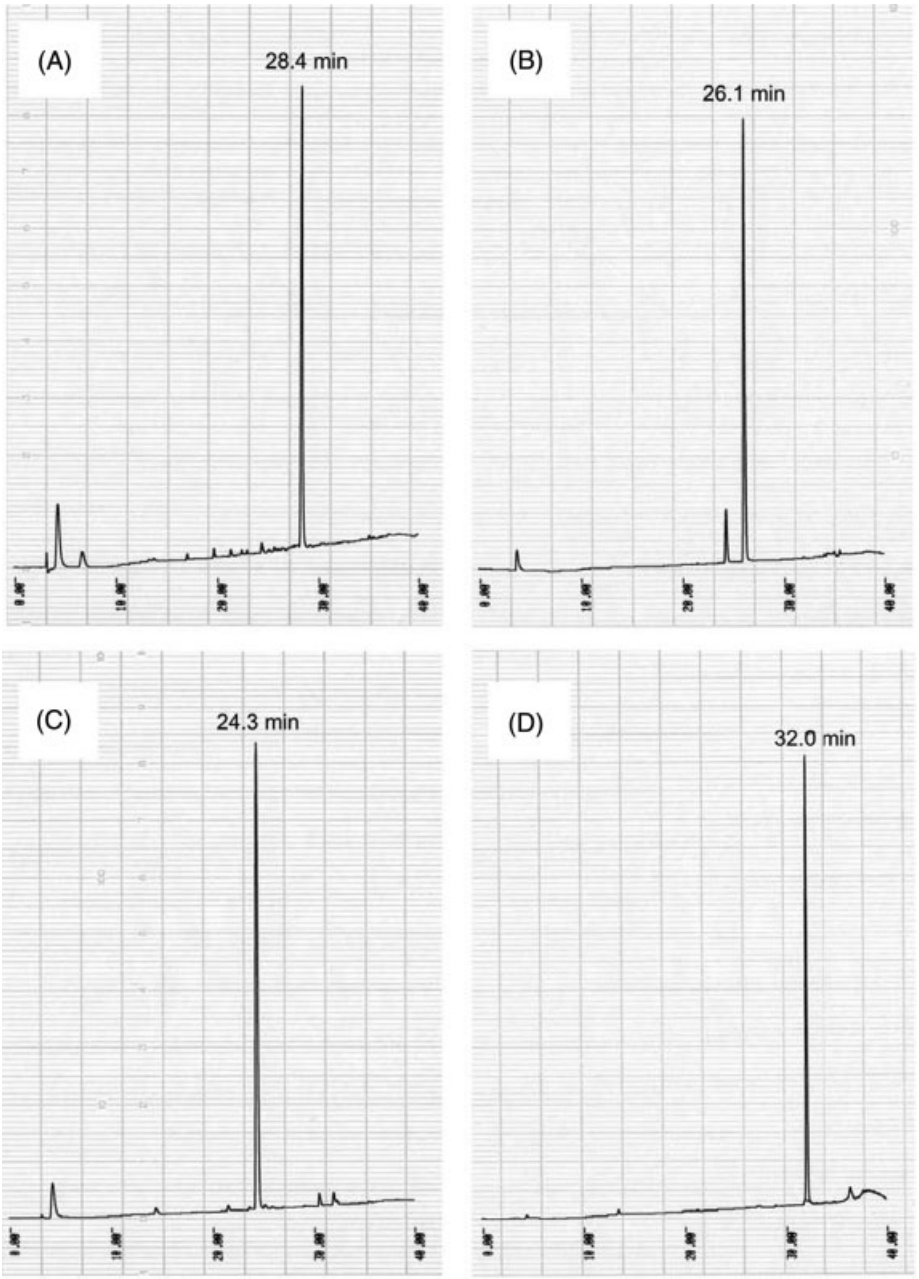

Figure 5. Analytical HPLC profiles of peptides obtained by in-water synthesis. (A) Boc-Phe-Leu-NH_2. (B) Boc-Gly-Phe-Leu-NH_2. (C) Boc-Gly-Gly-Phe-Leu-NH_2. (D) Boc-Tyr(*t*Bu)-Gly-Gly-Phe-Leu-NH_2. Elution was carried out for over 30 min at a flow rate of 1 ml/min with a linear gradient from 9 : 1 to 3 : 7 mixture of 0.05% aqueous TFA and 0.05% TFA in acetonitrile.

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