Research Article

Thermal Synthesis of Polypeptides from *N*-Butyloxycarbonyl Oligopeptides Containing Aspartyl Residue at C-Terminus

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The thermal reactions of amino acids have been investigated for pure organic synthesis, materials preparation in industry, and prebiotic chemistry. *N*-t-Butyloxycarbonyl aspartic acid (Boc-Asp) releases 2-butene and carbon dioxide upon heating without solvents. The resulting mixture of the free molten aspartic acid was dehydrated to give peptide bonds. This study describes the thermal reactions of *N*-t-butyloxycarbonyl peptides (Boc-Gly-L-Asp, Boc-L-Ala-L-Asp, Boc-L-Val-L-Asp, and Boc-Gly-Gly-L-Asp) having an aspartic residue at the carboxyl terminus. The peptides were deprotected upon heating at a constant temperature between 110 and 170°C for 1 to 24 h to afford polypeptides in which the average molecular weight reached 7800.

1. Introduction

Polypeptides [1] have been well investigated as protein model compounds [1–8]. Numerous reports on the methodology for the synthesis of polypeptides have been published [1–14]. The *N*-carboxyl- α -amino acid anhydride (NCA) method (1) [2, 3, 9, 10], polymerization of amino acids using active esters (2) [1, 4–7], solid-phase peptide synthesis (3) [8, 12], and the heating of amino acids (4) [13, 14] are typical examples. The NCA method (1) is suitable for making homopolypeptides and random copolypeptides but is not suitable for the synthesis of sequential copolyamino acid, which is more important for the build-up of functional polypeptides. Sequential polyamino acids have repetitive amino acid residues, in which the amino acid residues can be like $-(\text{Gly-Gly-Asp})_n-$. The active ester method (2) [1, 4–7] and solid phase synthesis (3) [8] are more suitable for the synthesis of sequential copolyamino acid. However, the problems of methods (2) and (3) are a long reaction time and the use of much solvent. In contrast, the synthesis of polyamino acid by heating a derivative of the amino acid (4) [13, 14] requires neither long reaction time nor solvents.

In previous papers [15, 16], we reported the synthesis of homopolypeptides [15] and random copolypeptides [16] upon heating of *N*-t-butyloxycarbonyl aspartic acid anhydride (Boc-Asp anhydride) and mixtures of Boc-L-Asp, anhydride, and Boc-Gly, Boc-L-Ala, or Boc-Val. In this paper, we report a trial for the synthesis of sequential copolypeptides by the heating of Boc-peptides instead of these anhydrides. As shown in Figures 1 and 2, Boc-peptides (**5a–d**) and Boc-L-Asp (**5e**) were prepared for heating under a stream of N_2 .

2. Materials and Methods

2.1. Instrumentation. A nuclear magnetic resonance (NMR) (JEOL FX-100 NMR system (JEOL, Tokyo, Japan)) was used for the collection of ^1H -NMR spectra. A Hitachi model 260-50 infrared (IR) spectrophotometer (Hitachi, Tokyo, Japan) was used for the collection of IR spectra. A Hitachi 200-10 spectrophotometer was used for spectrophotometry measurements.

A Jasco DIP-181 digital polarimeter (Jasco, Tokyo, Japan) was used for the measurement of the optical rotation of the peptide derivatives. A Hitachi 163 gas chromatograph

FIGURE 1: Preparation of *N*-*t*-butyloxycarbonyl-amino acid and peptide active esters. Boc_2O : di-*t*-butyl dicarbonate; NEt_3 : triethylamine; HONSu: *N*-hydroxysuccinimide; DCC: *N,N'*-dicyclohexylcarbodiimide.

FIGURE 2: Preparation of *N*-*t*-butyloxycarbonyl peptides containing aspartyl residue at C-terminus. TsOH: *p*-toluenesulfonic acid; TsO^- : *p*-toluenesulfonate; Boc_2 : di-*t*-butyl dicarbonate.

equipped with a chiral glass capillary column Chirasil-Val [17, 18] was used for the separation of the enantiomeric derivatives of amino acids. For thermal analysis, we used a Shimadzu DT-40 thermal analyzer (Shimadzu, Kyoto, Japan). A Jasco Trirotar-V as the flow pump and a Jasco UVDEC-100-IV spectrophotometer as the detector were used for the HPLC system equipped with a gel permeation column G-3000 PW (TSK, Yamaguchi, Japan). Analysis of the evolved gases from the thermal analyzer was performed with a Shimadzu GCMS-QP1000A.

2.2. Materials

2.2.1. Starting Materials and Reagents for the Preparation of Substrate Peptide Derivatives. Glycine (**1a**), L-alanine (**1b**), and L-valine (**1c**) were supplied by Nippon Rika Co., Ltd. (Tokyo, Japan). Glycylglycine (**1d**) and di-*t*-butyl dicarbonate (Boc_2O) were purchased from Peptide Institute, Inc. (Minoh-shi, Osaka, Japan). L-Aspartic acid (**1e**) and *N*-hydroxysuccinimide (HONSu) were purchased from Nacalai Tesque (Kyoto, Japan). *N,N'*-Dicyclohexylcarbodiimide

(DCC) was supplied by Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). Palladium on charcoal was purchased from Nippon Engelhard Ltd. (now N.E. CHEMCAT, Tokyo, Japan). Trifluoroacetic anhydride and triethylamine (NEt_3) were purchased from Tokyo Chemical Industries Co., Ltd. (Tokyo, Japan). Hydrochloric acid (6 M) for the hydrolysis of peptides and acetic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The 2-propanol solution containing 2.0 M hydrogen chloride was prepared by bubbling HCl into 2-propanol.

2.2.2. Preparation of Substrates

(1) *N*-*t*-Butyloxycarbonyl Amino Acids (**2a-d**)

Boc-Gly (2a**).** Glycine (7.51 g, 0.100 mol) was dissolved in an aqueous solution (150 mL) containing 0.100 mol NaOH, to which a 1,4-dioxane solution (150 mL) including di-*t*-butyl dicarbonate (24.0 g, 0.11) was added dropwise. After 2 h stirring, the reaction solution was evaporated in vacuo to an oily product, which was dissolved in 10% potassium

hydrogen sulfate to reach a pH of 2.5. The resulting solution was extracted with ethyl acetate (100 mL, three times). The extracts were combined, washed with brine, and dried with anhydrous MgSO_4 . The resulting ethyl acetate solution was evaporated in vacuo. The obtained precipitate was recrystallized with ethyl acetate and petroleum ether to give 10.1 g (yield 57%); the melting point (mp) was 91°C (in lit. [19], it is 94–95°C). $^1\text{H-NMR}$ (DMSO-D_6): δ 7.04 (t, J = 6.0 Hz, H, NH), 3.57 (d, J = 7.0 Hz, 2H, CH_2), 1.38 ppm (s, 9H, CH_3); IR (KBr, cm^{-1}): 3410, 3350, 3116 (NH), 1750 (COOH), 1671 (amide I), 1539 (amide II); elementary analysis: Calcd for $\text{C}_7\text{H}_{13}\text{NO}_4$: C, 47.99; H, 7.48; N, 8.00%. Found: C, 48.09; H, 7.58; N, 7.91%.

Other *N*-*t*-butyloxycarbonyl-amino acids were prepared in a similar manner. Their physical properties are detailed below.

Boc-L-Ala (2b). Yield 76%, mp 87–88°C (lit. [19] 83–8°C). $^1\text{H-NMR}$ (DMSO-D_6): δ 7.07 (d, J = 4 Hz, 1H, NH), 3.97 (q, J = 10 Hz, 1H, CH), 1.22 (d, J = 8.0 Hz, 3H, CH_3), 1.38 ppm (s, 9H, CH_3). IR (KBr, cm^{-1}): 3386 (NH), 1742 (COOH), 1690 (amide I), 1520 (amide II). Elementary analysis: Calcd for $\text{C}_8\text{H}_{15}\text{NO}_4$: C, 50.78; H, 7.99; N, 7.40%. Found: C, 50.78; H, 8.10; N, 7.27%. $[\alpha]^{15}_{\text{D}}$ –24.2 (c 1.30, acetic acid) (lit. $[\alpha]^{20}_{\text{D}}$ –27 (c 2.26, acetic acid) [19]).

Boc-L-Val (2c). Yield 93%, mp 78–79°C (lit. 80°C [19]). $^1\text{H-NMR}$ (DMSO-D_6): δ 6.90 (d, J = 9.0 Hz, 1H, NH), 3.80 (m, 1H, CH), 1.84–2.11 (m, 1H, CH_3), 1.39 (s, 9H, CH_3), 0.87 ppm (d, J = 4.0 Hz, 6H, CH_3). IR (KBr, cm^{-1}): 3312 (NH), 1740 (COOH), 1649 (amide I), 1500 (amide II). Elementary analysis: Calcd for $\text{C}_{10}\text{H}_{19}\text{NO}_4$: C, 55.28; H, 8.81; N, 6.45%. Found: C, 55.42; H, 8.86; N, 6.45%. $[\alpha]^{15}_{\text{D}}$ –5.1 (c 0.97, acetic acid) (lit. $[\alpha]^{20}_{\text{D}}$ –5.8 (c 1.2, acetic acid) [19]).

Boc-Gly-Gly (2d). Yield 73%, mp 138–139°C. $^1\text{H-NMR}$ (DMSO-D_6): δ 8.02 (t, J = 6.0 Hz, 1H, NH), 6.97 (q, J = 6.0 Hz, 1H, NH), 3.77 (d, J = 5.0 Hz, 3H, CH_2), 3.57 (d, J = 6.0 Hz, 2H, CH_2), 1.38 ppm (s, 9H, CH_3). IR (KBr, cm^{-1}): 3362 (NH), 1742, 1690 (COOH), 1618 (amide I), 1526 (amide II). Elementary analysis: Calcd for $\text{C}_9\text{H}_{16}\text{N}_2\text{O}_5$: C, 46.55; H, 6.94; N, 12.06%. Found: C, 46.70; H, 7.37; N, 12.02%.

(2) *N*-*t*-Butyloxycarbonyl-Amino Acid *N*-Hydroxysuccinimide (HONSu) Esters (3a–d)

Boc-Gly-ONSu (3a). *N*-*t*-Butyloxycarbonyl-glycine (23.73 g, 0.135 mol) and *N*-hydroxysuccinimide (HONSu) (17.26 g, 0.15 mol) were dissolved in ethyl acetate (410 mL). *N*, *N'*-dicyclohexylcarbodiimide (DCC) (34.05 g, 0.165 mol) dissolved in ethyl acetate (60 mL) was added to the cooled ethyl acetate solution at 0°C. The reaction mixture was stirred for about 36 h at 0°C. After the precipitate was filtered off, the obtained filtrate was evaporated in vacuo to give a colorless crystal, which was recrystallized with 2-propanol to give a colorless crystal (18.45 g, 50%), mp 155°C (lit. 168–170°C [19]). $^1\text{H-NMR}$ (DMSO-D_6): δ 7.48 (t, J = 6.5 Hz, 1H, NH), 4.09 (d, J = 6.0 Hz, 2H, CH_2), 2.81 (s, 4H, CH_2), 1.39 ppm (s, 9H, CH_3). IR (KBr, cm^{-1}): 3302 (NH), 1824, 1792 (ONSu), 1740

(ester). Elementary analysis: Calcd for $\text{C}_{11}\text{H}_{16}\text{N}_2\text{O}_6$: C, 48.53; H, 5.92; N, 10.29%. Found: C, 48.05; H, 5.90; N, 10.32%. By the similar manner, the other succinimide esters (3b–d) were prepared as follows.

Boc-L-Ala-ONSu (3b). 91%, mp 165–167°C (lit. 167°C [19]). $^1\text{H-NMR}$ (DMSO-D_6): δ 7.63 (d, J = 7.0 Hz, 1H, NH), 4.38 (q, J = 7.3 Hz, 1H, CH), 3.08 (s, 4H, CH_2), 1.40 (d, J = 6.0 Hz, 3H, CH_3), 1.39 ppm (s, 9H, CH_3). IR (KBr, cm^{-1}): 3296 (NH), 1827, 1792 (ONSu), 1742 (ester). Elementary analysis: Calcd for $\text{C}_{12}\text{H}_{18}\text{N}_2\text{O}_6$: C, 50.35; H, 6.34; N, 9.79%. Found: C, 50.52; H, 6.72; N, 9.73%. $[\alpha]^{15}_{\text{D}}$ –48.2 (c 2.16, 1,4-dioxane) (lit. $[\alpha]^{20}_{\text{D}}$ –49 (c 2, 1,4-dioxane) [19]).

Boc-L-Val-ONSu (3c). 85%, mp 131–132°C (lit. [19] 128–129°C). $^1\text{H-NMR}$ (DMSO-D_6): δ 7.57 (d, J = 7.0 Hz, 1H, NH), 4.23 (d, J = 5.0 Hz, 1H, CH), 2.81 (s, 4H, CH_2), 2.05–2.26 (m, 1H, CH), 1.41 (s, 9H, C CH_3), 1.00 ppm (d, J = 7.0 Hz, 6H, CH_3). IR (KBr, cm^{-1}): 3354 (NH), 1810, 1783 (ONSu), 1742 (ester), 1671 (amide I), 1539 (amide II). Elementary analysis: Calcd for $\text{C}_{14}\text{H}_{22}\text{N}_2\text{O}_6$: C, 53.49; H, 7.05; N, 8.91%. Found: C, 53.56; H, 7.19; N, 8.84%. $[\alpha]^{15}_{\text{D}}$ –23.4 (c 1.81, 1,4-dioxane) (lit. $[\alpha]^{20}_{\text{D}}$ –37.0 (c 2, 1,4-dioxane) [19]).

Boc-Gly-Gly-ONSu (3d). 71%, mp 164–165°C. $^1\text{H-NMR}$ (DMSO-D_6): δ 8.45 (t, J = 6.0 Hz, 1H, NH), 7.19 (t, J = 10 Hz, 1H, NH), 4.26 (d, J = 6.0 Hz, 2H, CH_2), 3.60 (d, J = 7.0 Hz, 2H, CH_2), 2.82 (s, 4H, CH_2), 1.39 ppm (s, 9H, CH_3). IR (KBr, cm^{-1}): 3416, 3300 (NH), 1825, 1792 (ONSu), 1742 (ester), 1702, 1665 (amide I), 1576, 1516 (amide II). Elementary analysis: Calcd for $\text{C}_{13}\text{H}_{19}\text{N}_3\text{O}_7$: C, 47.42; H, 5.82; N, 12.76%. Found: C, 47.50; H, 5.88; N, 12.63%.

(3) *N*-*t*-Butyloxycarbonyl-Peptides (5a–d)

TsOH L-Asp(OBzl)₂ (4). L-Aspartic acid (1e) (19.97 g, 0.150 mol), *p*-toluenesulfonic acid monohydrate 1 (29.10 g, 0.153 mol), benzyl alcohol (300 mL), and benzene (300 mL) were mixed in a three-necked flask that was connected to a Dean-Stark apparatus. The esterification reaction was carried out by refluxing the reaction mixture and removing water in the flask for 7 days. The cooled reaction solution was evaporated in vacuo to give a colorless solid, which was recrystallized with chloroform and petroleum ether. Yield 49.8 g (68%), mp 157–158°C (lit. 159–160°C [19]). $^1\text{H-NMR}$ (DMSO-D_6): δ 8.54 (s, 1H, SO_3H), 7.51 (d, J = 10 Hz, 2H, ArH), 7.36 (s, 10H, ArH), 7.11 (d, J = 9.0 Hz, 2H, ArH), 5.14 (d, J = 8.0 Hz, 2H, NH_2), 4.50 (t, J = 5.5 Hz, 1H, CH), 3.40 (s, 4H, CH_2), 3.05 (d, J = 8.0 Hz, 2H, CH_2), 2.28 ppm (s, 3H, CH_3). IR (KBr, cm^{-1}): 3042 (NH_3), 1758, 1738, 1127 (ester), 1185 (– SO_3), 814, 739, 685. Elementary analysis: Calcd for $\text{C}_{25}\text{H}_{27}\text{NO}_7\text{S}$: C, 61.84; H, 5.61; N, 2.89%. Found: C, 61.94; H, 5.63; N, 2.85%. $[\alpha]^{15}_{\text{D}}$ –1.04 (c 2.21, methanol) (lit. $[\alpha]^{25}_{\text{D}}$ +1.0 (c 1, methanol) [19]).

Boc-Gly-L-Asp (5a). L-Aspartic acid (1e) (7.98 g, 0.060 mol) and triethylamine (NEt_3) (12.14 g, 0.12 mol) were dissolved in distilled water (250 mL), and Boc-Gly-ONSu (13.61 g, 0.050 mol) dissolved in 1,4-dioxane (250 mL) for 4 h was

added to the solution. The resulting solution, which was acidified with 10% KHSO_4 to give a pH of 2.5, was extracted with ethyl acetate (100 mL, three times). The combined extracts were washed with brine and dried with anhydrous MgSO_4 . The filtrated solution was evaporated in vacuo to give a colorless crystal that was recrystallized with ethyl acetate and petroleum ether to yield 10.17 g (70%); mp 130°C; $^1\text{H-NMR}$ (DMSO-D_6): δ 8.02 (d, J = 8.0 Hz, 1H, NH), 6.96 (t, J = 4.0 Hz, 1H, NH), 4.56 (t, J = 10 Hz, 1H, CH), 3.56 (q, J = 2.0 Hz, 2H, CH_2), 2.66 (d, J = 6.0 Hz, 2H, CH_2), 1.38 ppm (s, 9H, CH_3). IR (KBr, cm^{-1}): 3432, 3340 (NH), 1756, 1711 (COOH), 1659 (amide I), 1547 (amide II). Elementary analysis: Calcd for $\text{C}_{11}\text{H}_{18}\text{N}_2\text{O}_7$: C, 45.52; H, 6.25; N, 9.65%. Found: C, 45.43; H, 6.35; N, 9.48%. $[\alpha]^{15}_{\text{D}}$ +20.7 (c 1.06, acetic acid).

Boc-L-Ala-L-Asp (**5b**). *Boc-L-Ala-ONSu* (**3b**) (0.020 mol) was coupled with *TsOH L-Asp* (*OBzl*)₂ (**4**) in dichloromethane to yield *Boc-L-Ala-L-Asp* (*OBzl*)₂ of 84%, which was hydrogenolyzed over 5% Pd on charcoal to yield *Boc-L-Ala-L-Asp*. Yield: 3.21 g, 70%, 85°C (decomposed). $^1\text{H-NMR}$ (DMSO-D_6): δ 7.97 (d, J = 8.0 Hz, 1H, NH), 6.94 (d, J = 7.0 Hz, 1H, NH), 4.50 (t, J = 10 Hz, 1H, CH), 3.95 (q, J = 7.0 Hz, 2H, CH), 2.63 (d, J = 6.0 Hz, 2H, CH_2), 1.37 (s, 9H, CH_3), 1.17 ppm (d, J = 7.0 Hz, 3H, CH_3). IR (KBr, cm^{-1}): 3432, 3340 (NH), 1756, 1711 (COOH), 1659 (amide I), 1547 (amide II). Elementary analysis: Calcd for $\text{C}_{12}\text{H}_{20}\text{N}_2\text{O}_7$ 0.45THF: C, 49.22; H, 7.06; N, 8.32%. Found: C, 48.98; H, 7.09; N, 8.07%. $[\alpha]^{15}_{\text{D}}$ +3.81 (c 0.972, acetic acid). Other substrates (**5c**, **5d**) were prepared in a similar manner.

Boc-L-Val-L-Asp (**5c**). 72%, mp 85°C (decompose). $^1\text{H-NMR}$ (DMSO-D_6): δ 8.10 (d, J = 7.0 Hz, 1H, NH), 6.65 (d, J = 9.0 Hz, 1H, NH), 4.53 (t, J = 10 Hz, 1H, CH), 3.83 (d, J = 2.0 Hz, 1H, CH), 2.65 (d, J = 9.0 Hz, 2H, CH_2), 1.70–1.83 (m, 1H, CH), 1.38 (s, 9H, CH_3), 0.86 (d, J = 6.0 Hz, 3H, CH_3), 0.80 ppm (d, J = 6.0 Hz, 3H, CH_3). IR (KBr, cm^{-1}): 3432, 3340 (NH), 1756, 1711 (COOH), 1659 (amide I), 1547 (amide II). Elementary analysis: Calcd for $\text{C}_{14}\text{H}_{24}\text{N}_2\text{O}_7$ 0.45THF: C, 52.02; H, 7.63; N, 7.68%. Found: C, 52.03; H, 7.60; N, 7.41%. $[\alpha]^{15}_{\text{D}}$ -5.67 (c 1.04, acetic acid).

Boc-Gly-Gly-L-Asp (**5d**). 97%, mp 82–92°C. $^1\text{H-NMR}$ (DMSO-D_6): δ 8.20 (d, J = 8.0 Hz, 1H, NH), 8.02 (t, J = 2.5 Hz, 1H, NH), 7.10 (t, J = 11 Hz, 1H, NH), 4.55 (t, J = 10 Hz, 1H, CH), 3.75 (d, J = 5.0 Hz, 2H, CH_2), 3.57 (d, J = 6.0 Hz, 2H, CH_2), 2.64 (d, J = 0.2 Hz, 2H, CH_2), 1.39 ppm (s, 9H, CH_3). IR (KBr, cm^{-1}): 3354 (NH), 1725 (COOH), 1657 (amide I), 1535 (amide II). Elementary analysis: Calcd for $\text{C}_{13}\text{H}_{21}\text{N}_3\text{O}_8$ 0.50THF: C, 46.99; H, 6.57; N, 10.96%. Found: C, 47.05; H, 6.56; N, 10.85%. $[\alpha]^{15}_{\text{D}}$ +16.2 (c 1.04, acetic acid).

Boc-L-Asp (**5e**). 60%, mp 116–118°C (lit. [19] 118–119°C). $^1\text{H-NMR}$ (DMSO-D_6): δ 7.02 (d, J = 8.0 Hz, 1H, NH), 4.26 (q, J = 7.3, 15 Hz, 1H, CH), 2.63 (d, J = 4.0 Hz, 2H, CH_2), 1.38 ppm (s, 9H, CH_3). IR (KBr, cm^{-1}): 3358 (NH), 1720 (COOH), 1694 (amide I), 1539 (amide II). Elementary analysis: Calcd for $\text{C}_9\text{H}_{15}\text{NO}_6$: C, 46.35; H, 6.48; N, 6.01%. Found: C, 46.37; H, 6.59; N, 5.85%. $[\alpha]^{15}_{\text{D}}$ -4.5 (c 1.15, methanol) (lit. $[\alpha]^{20}_{\text{D}}$ -6.4 (c 1, methanol) [19]).

2.3. Thermal Analysis. A small scale of the reaction was performed in the thermal analyzer using the substrate under an N_2 stream to monitor the thermal gravimetry (TG) and differential thermal analysis (DTA); *Boc-Gly-L-Asp* (3.30 mg) was heated from 50°C to 175°C at a rate of 4.7°C per min. The gas mixture that formed during the heating reaction was directly derived into a mass spectrometer (Shimadzu QP-1000A). The gases were determined by selective ion monitoring as CO_2 (m/z = 44), isobutene (m/z = 56), and water (m/z = 18).

2.4. Thermal Reaction. *N-Boc-dipeptides* (**5a–5c**, 0.5 mmol) and *N-Boc-tripeptide* (**5d**, 0.3 mmol), which were put into different Pyrex test tubes (165 mm $\times$ 18 internal diameter (i.d.)), were kept for 5 min under N_2 flow and then heated in an oil bath controlled at a constant temperature under N_2 flow. After the heating reactions, the reaction mixtures in the test tubes were kept under vacuum for 24 h at room temperature. The weight decrease and IR spectra of the resulting samples were measured.

2.5. Gel Filtration. The overall amount of each reaction mixture after the heating reaction was dissolved in 5 mL of 0.5 M acetic acid and the resulting solution was loaded onto a gel permeation chromatograph (910 mm $\times$ 15 mm i.d.). During the elution with 0.5 M acetic acid, the eluate was collected by 3 mL fractions in 95 test tubes. The ultraviolet (UV) absorption of each collected fraction was recorded at 230 nm. 3 M acetic acid was used only for the reaction mixture using *Boc-L-Val-L-Asp* (**5c**) instead of 0.5 M acetic acid.

2.6. Molecular Weight Estimation. The collected fractions were tested for ninhydrin using thin-layer chromatography in a developing solvent: 1-butanol–acetic acid–water (4:1:2 (v/v)). Ninhydrin-negative fractions were combined as higher molecular weight fractions, and ninhydrin-positive fractions were combined as lower molecular weight fractions, which showed not a spot but a tailed area with R_f values in the range from 0 to ca. 0.4. The higher molecular weight fractions were lyophilized to afford an amorphous peptide powder, of which a part (1 to 2 mg) was dissolved in 0.1 M sodium phosphate buffer (NaH_2PO_4 – Na_2HPO_4 , pH 6.9). A part (20 μL) of the sample solution was mixed with 20 μL blue dextran solution (1.5 mg/mL 0.1 M sodium phosphate buffer pH 6.9) and was injected into the TSK gel G-3000 PW in an HPLC system. The retention of the sample on the chromatogram was compared with the calibration line that was prepared with several retention times of proteins of known molecular weight.

2.7. Hydrolysis of Polypeptides and Amino Acid Analysis. About 2 mg of each higher molecular weight fraction described in Section 2.5 was mixed with 2 mL of 6 M-HCl in a glass test tube that was sealed under vacuum. The sealed glass was heated to 110°C and held for 8 h, which was enough for the complete hydrolysis of the sample peptide. The resulting hydrolysate was analyzed by means of the automatic amino acid analyzer.

FIGURE 3: Thermal analysis of Boc-Gly-L-Asp (5a). Heating rate: 4.7°C/min; carrier gas: He, 50 mL/min; AMP range: DTA: 200 μ V; TG: 10 mg on a Pt cell (2.65 mm $\times$ 5.40 i.d.).

2.8. D/L Ratio Analysis. 1 mL of each acid-hydrolysate described above was put in a glass vial and kept under a vacuum. The obtained constant weight of the sample was esterified with 2 mL of 2-propanol solution containing 1.5 M HCl and then with trifluoroacetic anhydride to give *N*-trifluoro-amino acid 2-propyl ester (*N*-TFA-AA-O-2-Pr).

3. Results and Discussion

3.1. Weight Decrease and Ion Monitoring during the Heating Reactions of Boc-Peptides and Boc-L-Asp. The charts of DTA and TG for Boc-Gly-L-Asp (5a) are shown in Figure 3.

Although the DTA line curved gently down during the time the TA line was flat, both lines go rapidly down after the temperature of 115°C was reached, which corresponded with a temperature a little lower than the mp (130°C). The thermal absorption on the DTA line continued and became almost flat at 175°C. The TG line also became flat and the total decrease in weight was 40.7% at 175°C. This ratio corresponds to the theoretical weight decrease after the decomposition of the Boc group (34.5%) and dehydration (6.2%) between free peptides from the initial weight of the Boc-Gly-L-Asp compound. To monitor the evolved gases during the heating reaction of Boc-Gly-L-Asp, the thermal analyzer was directly connected to a mass spectrometer (MS). The MS records showed 2-butene ($m/z = 56$), CO₂ ($m/z = 44$), and water ($m/z = 18$) at temperature conditions higher than about 110°C (Figure 4). Monitoring water generation on the line, there were two peaks of water. The peak at the lower temperatures seems to be the water generation during the peptide bond formation between deprotected dipeptides. Another peak at the higher temperatures is supposed to be the water generation during the imide formation.

As a control experiment, the thermal analysis of Boc-L-Asp was conducted during monitoring the generated gases by mass spectrometry as shown in Figures 5 and 6.

The curves of TA and DTA of Boc-L-Asp in Figure 5 show almost flat lines (the first flat) from room temperature to 100°C and then a rapid decrease of weight on TA plus an energy absorption peak on DTA. In the range of higher temperatures, the curve of DTA became flat again (the second flat) at 180°C and rapidly falls down and then is flat (the third flat) at 216°C. The weight decrease from the first to the second flat corresponds with 49.2% of the initial weight of Boc-Gly-L-Asp. The theoretical weight decrease is caused almost totally (50.7%) by 2-butene (24.1%), CO₂ (18.9%), and water (7.7%). These gases were monitored in the lower temperature range up to 180°C (Figure 6). The weight decrease (11.5%) from the second to the third flat may include generation of water (7.7%) by imide formation and a little decomposition. The temperature range of the weight decrease (11.5%) corresponded to the energy absorption peak on the DTA curve (Figure 5) and the second peak of water generation in Figure 6.

Compared with the thermal analyses of Boc-Gly-L-Asp and Boc-L-Asp, the dehydration in the thermal reaction of Boc-Gly-L-Asp might proceed at temperatures lower than those of the thermal reaction of Boc-L-Asp [15, 20], which did not produce peptides but amino acid at lower temperatures. A similar feature was observed for Boc-DL-Asp [20] and Boc-L-asparagine (Boc-L-Asn) [21]. The latter case released ammonia as well as 2-butene, CO₂, and water.

3.2. Thermal Reactions of *N*-Boc-Peptides (5a–5d). Table 1 shows the weight decrease during the heating reactions of *N*-Boc-peptides under different conditions. The heating reaction of Boc-Gly-L-Asp under these conditions gave about 37% to 46% weight decrease, almost equal to the total release (41%) of 2-butene, CO₂, and water. Boc-L-Ala-L-Asp, Boc-L-Val-L-Asp, and Boc-Gly-Gly-L-Asp theoretically release 2-butene, CO₂, and water at the ratio of 39%, 34%, and 29%, respectively. At temperatures below 130°C, these compounds showed a lower decrease than the theoretical decrease. The reactions of these substrates at 130°C for 4 h are enough for the complete release of Boc and water.

3.3. Gel Filtration of Reaction Mixtures. The thermal reaction products were loaded onto a gel permeation chromatograph. A constant volume (ca. 3 mL) of eluate was added to the test tubes. Figures 7–9 show the absorbance of each collected eluate at 230 nm plotted against the test tube number, which was proportional to the total volume of elution after the start of loading.

Figure 7 shows chromatograms of the reaction mixtures of Boc-Gly-L-Asp at 130°C for varying reaction times. Two peaks appear in each chromatogram. The first and the second peaks included the higher and lower molecular weight fraction, respectively. The first peak is revealed faster with the reaction time proceeded until 8 h reaction (d) and is revealed later after 8 h reaction. The results suggested that the polymerization reaction mainly proceeded during 8 h and degradation proceeded mainly after 8 h.

Figure 8 shows the chromatograms of the heating reaction mixtures of Boc-Gly-L-Asp (5a) at varying temperatures. Comparing the first peaks of chromatograms ((a) and (b)) at

FIGURE 4: Thermal release patterns of ions for compounds from Boc-Gly-L-Asp (**5a**) by DTA/TG-MS analysis. Ion source: EI, 70 eV; temperature: 250°C. Detection: EI range, m/z = 17 to 100; interval, 1.5 s; gain, 2.5; pipe heater: 280°C. GC oven: 150°C; injection: 200°C.

FIGURE 5: Thermal analysis of Boc-L-Asp (**5e**). Heating rate: 4.7°C/min; carrier gas: He, 50 mL/min; AMP range: DTA (differential thermal analysis): 200 μ V, TG (thermogravimetry): 10 mg on a platinum cell (2.65 mm $\times$ 5.40 i.d.).

FIGURE 6: Thermal release patterns of ions for compounds from Boc-L-Asp (**5a**) by DTA/TG-MS analysis. Ion source: EI, 70 eV; temperature: 250°C. Detection: EI range, m/z = 17 to 100; interval, 1.5 sec; gain, 2.5. Pipe heater: 280°C. GC oven: 150°C; injection: 200°C.

TABLE 1: Weight decrease in the heating reaction of substrate peptides.

Substrate	Temperature/°C	Reaction time/h	Weight/mg		Weight decrease	
			Initial	Later	mg	%
Boc-Gly-L-Asp (5a)	110	24	145	88	57	39
	120	24	145	89	56	39
	130	1	145	92	53	37
	130	2	143	86	57	40
	130	4	146	86	60	41
	130	8	146	83	63	43
	130	16	148	84	64	43
	130	24	145	84	61	42
	150	2	146	81	65	45
	170	2	147	80	67	46
Boc-L-Ala-L-Asp (5b)	120	1	161	120	41	25
	120	2	151	117	34	23
	120	4	162	121	41	25
	130	1	146	92	54	37
	130	2	174	100	74	43
	130	4	179	99	80	45
Boc-L-Val-L-Asp (5c)	110	1	164	160	4	2
	110	2	160	132	28	18
	110	4	163	126	37	23
	120	1	161	134	27	17
	120	2	186	160	26	14
	120	4	169	115	54	32
	130	1	170	141	29	17
	130	2	165	116	49	30
	130	4	166	109	57	34
Boc-Gly-Gly-L-Asp (5d)	120	1	113	107	6	5
	120	2	107	99	8	7
	120	4	110	74	36	33
	130	1	109	80	29	27
	130	2	104	69	35	34
	130	4	104	71	33	32

different temperatures, the first peak on (b) (120°C, 24 h) was revealed faster than (a) (110°C, 24 h). The result suggested that the condition at 120°C, 24 h, was better for polymerization than 110°C, 24 h. Comparing the first peaks of chromatograms ((c) and (d)) at different temperatures, the first peak of (c) (150°C, 2 h) was revealed faster than (a) (170°C, 2 h). The result suggested that the condition at 150°C, 2 h, was better for polymerization than 170°C, 2 h. A high temperature, such as 170°C, fostered the degradation of polymers generated from the substrate Boc-Gly-L-Asp (5a).

The chromatograms of the reaction mixture using other substrates (5b–d) are shown in Figure 9. The chromatograms of 5b and 5c showed two peaks, although the chromatogram of the reaction mixture of 5c shows a not-so-clear second peak. This might have been caused by the use of an eluate with a higher concentration of acetic acid (3 M) which had stronger absorption than 0.5 M acetic acid at 230 nm but could enable the reaction mixture to dissolve in it.

The IR spectrum (Figure 10) of the higher molecular weight products shows the absorption of typical acidic polypeptides: amide I, amide II, -NH, and -COOH groups.

3.4. Estimation of the Molecular Weight of the Higher Molecular Weight Fraction. The molecular weight of the higher molecular weight fractions was estimated with the calibration curve (Figure 11) that shows the relationship between the common logarithm and the ratio V_e/V_0 between the elution volume and the void volume. Table 2 shows the values V_e/V_0 for the higher molecular weight fractions at varying reaction times and temperatures.

The estimated molecular weight was almost comparable with the position of the first peak on the chromatograms of Figures 7–9. The samples from the reaction of Boc-Gly-L-Asp for 24 h gave the following values: 4400, 7000, and 4600 Da for 110, 120, and 130°C, respectively. A proper combination of the reaction conditions gave the highest

FIGURE 7: Gel permeation chromatograms of the reaction mixtures of Boc-Gly-L-Asp at 130°C for varying reaction times: (a) 1 h; (b) 2 h; (c) 4 h; (d) 8 h; (e) 16 h; and (f) 24 h. Solid phase: Sephadex G-25F. Eluate: 0.5 M acetic acid. Volume per test tube: 3 mL. fa: ninthyrin-negative, higher molecular weight fractions; fb: ninthyrin-positive lower molecular weight fractions.

FIGURE 8: Gel permeation chromatograms of the reaction mixtures of Boc-Gly-L-Asp (5a) at varying temperatures and times: (a) 110°C, 24 h; (b) 120°C, 24 h; (c) 150°C, 2 h; and (d) 170°C, 2 h. Solid phase: Sephadex G-25F. Eluate: 0.5 M acetic acid. Volume per a test tube: 3 mL. fa: ninthrin-negative, higher molecular weight fractions; fb: ninthrin-positive lower molecular weight fractions.

molecular weight, and a temperature that was too high gave a lower molecular weight because of enhanced degradation. The effect of reaction time at the same temperature could be seen in detail in the reactions at 130°C. The shorter reaction times gave a lower molecular weight but it was higher with reaction time (2800, 4100, and 6900 for 1, 2, and 4 h, resp.); the medium reaction time gave the highest molecular weight: 7800 for 8 h; the longer reaction time gave a lower molecular weight (5400 and 4600 Da for 16 and 24 h, resp.) again. The other substrates resulted in a similar tendency as for substrate 5a.

3.5. Amino Acid Composition and D/L Ratio of the Residue in the Higher Molecular Weight Fraction. The amino acid composition and the D/L ratio in the acid-hydrolysate of the higher molecular weight fractions are listed in Table 3.

The amino acid compositions were almost even at the level of the theoretical value in the hydrolysates obtained with the higher molecular weight fractions of the Boc-dipeptides at lower temperatures and for shorter reaction times. However, the hydrolysates of the obtained higher molecular weight fractions at the higher temperature and longer reaction times gave higher compositions for amino

FIGURE 9: Gel permeation chromatograms of the reaction mixtures of peptide derivatives under different conditions. (a) Boc-L-Ala-L-Asp, 130°C, 1 h; (b) Boc-L-Val-L-Asp, 130°C, 2 h; and (c) 120°C, 4 h. Solid phase: Sephadex G-25F. Eluate: 0.5 M acetic acid. Volume per a test tube: 3 mL. fa: ninhydrin-negative, higher molecular weight fractions; fb: ninhydrin-positive lower molecular weight fractions.

acids other than aspartic acid, because the latter is more labile to decomposition at higher temperatures than other amino acids. The hydrolysates of the product formed a tripeptide derivative: Boc-Gly-Gly-L-Asp gave Gly and Asp with the ratio of 2:1. The amino acid recovery after hydrolysis was from 76% to almost the quantitative amount.

Regarding the D/L ratio, the molar ratio of the D-isomer to L-isomer of aspartic acid was higher in the hydrolysates of the products with the higher temperature and the longer reaction. The D/L ratio from the products was 0.142 at 130°C for 24 h, 0.189 at 150°C for 2 h, and 0.596 at 170°C for 2 h. The D/L ratios of other amino acids were lower than that for aspartic acid. The existence of D-isomers may be explained by racemization [22] of L-aspartic acid or epimerization of L-aspartyl residues in the polypeptides. L-Aspartic acid

undergoes the fastest racemization and epimerization of the all proteinous amino acids, because particularly aspartic residue forms imide structure which fosters the epimerization [23–26]. However, if some better reaction conditions were selected, such racemization and epimerization could be minimized to be lower than 10%. In contrast, racemization and epimerization of Asp residue might proceed in the range of 1% to 2% during 8 h hydrolysis at 110°C. The actual D/L ratio in the polypeptides must be 1% to 2% lower than the results in Table 3.

3.6. Analysis of the Lower Molecular Weight Fractions. HPLC results of the heating reaction mixtures of Boc-dipeptides (5a–c) gave two peaks on the chromatogram by means of G-3000PM, whereas the Boc-tripeptide (Boc-Gly-Gly-L-Asp:

FIGURE 10: IR spectrum of the higher molecular weight fraction of the reaction mixture obtained from Boc-Gly-L-Asp at 130°C for 8 h.

FIGURE 11: Calibration curve of the molecular weight of peptides using HPLC with a TSK-GEL G-3000PW (300 mm $\times$ 7.5 mm i.d.). Eluent: 0.1 M sodium phosphate buffer, pH 6.9; flow rate: 0.7 mL/min; detection: UV 230 nm. V_e : volume (mL) of elution for each sample; V_0 : elution volume of blue dextran as void volume (mL).

5d) gave only one (Figure 12: (a) Boc-L-Val-L-Asp (**5c**) and (b) Boc-Gly-Gly-L-Asp (**5d**)).

Chromatogram (a) gave only one peak, but (b) gave two peaks, in which the longer retention time peak was caused by the lower molecular weight products. The IR spectrum (Figure 12) of the lower molecular weight fraction from

Boc-L-Val-L-Asp showed absorption for the groups of Amide I (1663 cm^{-1}) and -COOH (1717 cm^{-1}) or imide and the IR spectrum of Boc-Gly-Gly-L-Asp showed Amide II, as well as -COOH , and Amide I (1545 , 1717 , and 1663 cm^{-1} , resp.). As the lower molecular weight fraction from the reaction mixture of Boc-L-Val-L-Asp (**5c**) has -COOH and amide groups but

TABLE 2: Estimated molecular weight of the higher molecular weight fraction.

Substrate	Temperature/°C	Reaction time/h	V_e/V_0^a	Molecular weight/Da	Number (n) of residues (AA-Asp) n
Boc-Gly-L-Asap (5a)	110	24	1.395	4400	26
	120	24	1.362	7000	41
	130	1	1.423	2800	16
	130	2	1.397	4100	24
	130	4	1.363	6900	40
	130	8	1.354	7800	45
	130	16	1.381	5400	31
	130	24	1.389	4600	23
	150	2	1.430	2500	15
	170	2	1.468	1400	8
Boc-L-Ala-L-Asp (5b)	130	1	1.466	1500	8
	130	2 ^b	1.421	2900	16
	130	4 ^b	1.382	5200	28
Boc-L-Val-L-Asp (5c)	120	4 ^b	1.447	2000	9
	130	2	1.441	2200	10
	130	4 ^b	1.393	4200	20
Boc-Gly-Gly-L-Asp (5d)	120	4	1.354	7800	34
	120	4 ^b	1.404	3700	16
	130	2 ^b	1.402	4000	17
	130	4 ^b	1.385	5000	21

^a V_e : volume of elution; V_0 : void volume; ^bintact reaction mixture without gel filtration.

TABLE 3: Amino acid composition and D/L ratio of hydrolysates of the higher molecular weight fractions.

Substrate	Temperature/°C	Reaction time/h	Mw/Da	AA composition		D/L ratio		AA recovery/%
				AA	Asp	AA	Asp	
Boc-Gly-L-Asp (5a)	110	24	4400	1.11	1.00	—	0.064	95
	120	24	7000	0.98	1.00	—	0.061	104
	130	1	2800	1.10	1.00	—	0.058	87
	130	2	4100	1.08	1.00	—	0.072	76
	130	4	6900	1.10	1.00	—	0.087	93
	130	8	7800	1.10	1.00	—	0.098	88
	130	16	5400	1.12	1.00	—	0.122	84
	130	24	4600	1.09	1.00	—	0.142	83
	150	2	2500	1.11	1.00	—	0.189	100
	170	2	1400	1.13	1.00	—	0.596	100
Boc-L-Ala-L-Asp (5b)	130	1	1500	1.00	1.00	0.042	0.067	88
Boc-L-Val-L-Asp (5c)	130	2	2200	0.94	1.00	0.017	0.099	78
Boc-Gly-Gly-L-Asp (5d)	120	4	7800	1.95	1.00	—	0.059	102

not the linear peptide, the fractions are suggested to include the structure of 2,5-diketopiperazine (DKP) [27] or a kind of imide [20] structure.

3.7. Postulated Mechanism of the Thermal Reaction of Boc-Peptides. From the many features of the thermal reactions of substrate Boc-peptides and the products described above,

we propose the reaction mechanism of the thermal reaction depicted in Figure 14.

There might be three reaction intermediates, **6**, **7**, and **8**, from substrates **5b–d** to polypeptide **9**. Intermediate **6** may be produced by releasing 2-butene and carbon dioxide first. Intermediate **7** may be produced by releasing water first. Intermediate **8** may be produced from intermediates **6** and **7**

FIGURE 12: Chromatograms of the crude products obtained from Boc-Gly-Gly-L-Asp (a) at 120°C for 4 h and Boc-L-Val-L-Asp (b) at 130°C for 2 h in the heating reactions using an HPLC equipped with a TSK-GEL G-3000PW (300 mm $\times$ 7.5 mm i.d.). Eluent: 0.1 M sodium phosphate buffer, pH 6.9; flow rate: 0.7 mL/min; detection: UV 230 nm.

or from substrate **5a–d** directly. The actual reaction mixture can include the three intermediates (**6**, **7**, and **8**), because the analysis of the gases directly injected from thermal reaction to the mass spectrometer indicated that the three gases continuously evolved at the same temperature, although the order is CO₂ plus water and then 2-butene (Figures 3 and 4). Therefore, intermediates **6** and **8** can yield polypeptide **9**. Intermediate **6** would directly dehydrate between an amino group and a carboxy group to yield a peptide bond. Intermediate **8** would be attacked by an amino group to open its anhydride group and form two kinds of peptide bond that are linked using the α - and β -carboxy groups; and then complete dehydration makes imide (**10**) bonds in polypeptides (**9**).

A part of dipeptide derivatives (**5a–c**) form six-membered ring compounds (2,5-diketopiperazine: DKP (**11**) [27]), which may not polymerize anymore. The existence of DKP in the lower molecular weight fractions can be supported by the IR spectrum (B) (Figure 13), which has the absorbance of DKP (1663 cm⁻¹) and carboxyl group of -COOH (1710 cm⁻¹). Another estimated structure showing absorbance at 1710 cm⁻¹ may be a tetrapeptide imide structure like compound **10** ($n = 1$; $q = 1$), which could form by coupling of two dipeptides, for instance, Val-Asp from **5c** in Figure 14. However, the anhydride structure of compound **10** would have partly opened by hydrolysis during chromatography. If the tetrapeptide imide structure cyclized from head to tail, the resulting product would have two five-membered cyclic imides and a twelve-membered cyclic

FIGURE 13: IR spectra of the lower molecular weight fractions obtained from Boc-Gly-Gly-L-Asp (A) at 120°C for 4 h and Boc-L-Val-L-Asp (B) at 130°C for 2 h in the reaction heating reactions.

peptide. Therefore, the lower molecular weight fraction may include DKP and the tetrapeptide imide structure. Further

FIGURE 14: Postulated mechanism of the thermal reaction of Boc-peptides (5a-d). $n = 1$ for 5a-c; $n = 2$ for 5d. m , n , o , p , and q : natural number (0, 1, 2, 3, ...).

FIGURE 15: Postulated branched structures from Boc-peptides upon heating. $n = 1$ for 5a-c; $n = 2$ for 5d. n and p : natural number (1, 2, 3, ...).

study of isolation of these compounds will give proof of the hypothesis.

The results in this paper reveal that the heating reactions of N-Boc-oligopeptides gave higher molecular peptides, which have almost the same amino acid compositions as their starting substrates.

The results suggest that the polypeptide products have a sequential structure but it is not established clearly. There may be some cases to yield branched polypeptides, which can be made during the reactions of the imide structure with the deprotected oligopeptides as shown in Figure 15. Since the chemical structure is very complexed, further research may be needed to clarify the details in the future research.

4. Conclusions

This investigation has demonstrated the first simple successive heating synthesis of copolypeptides by using peptide derivatives. In the thermal reactions, Boc-peptides melted, released a protecting group, and dehydrated to polypeptides. The mechanism was supported by thermal analysis accompanied by mass spectrometry. The holding amino acid residues in the polyamino acid structure were supported by amino acid analysis. The D/L ratio was suppressed to be below 10% at lower temperatures. These results suggested that the thermal reaction using Boc-peptides might be useful for producing sequential polypeptides. The sequential structure that is not proven should be clarified in the future research.

Disclosure

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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