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Thermal Synthesis of Polypeptides from *N*-*t*-Butyloxycarbonyl-tripeptide Derivatives without Reactive Side Chains

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The thermal reactions of *N*-*t*-butyloxycarbonyl-aspartic acid (Boc-Asp-OH) and some *N*-*t*-butyloxycarbonyl derivatives of peptides in which the *C*-terminal is an aspartic residue as a reactive residue in Boc-Gly-Gly-L-Asp-OH are known. The two carboxy groups at *C*-terminal are believed to form an anhydride or give peptide bonds directly. This research reports the thermal reactions of *N*-Boc-tripeptide derivatives such as Boc-Pro-Pro-Gly-OH, Boc-Pro-Pro-Gly-NH_2, Boc-Pro-Pro-Gly-OCH_3 and Boc-Ala- Ala-Ala-OH with a carboxy group at *C*-terminal. Thermal reactions were carried out at a constant temperature near their melting points for 1 to 24 h to afford polypeptides whose average molecular weight reached 2,500 Da.

Keywords: Thermal reaction, Sequential peptides, *N*-Boc-tripeptides, Proline, Alanine.

INTRODUCTION

Peptide structures with repetitive sequences have been found in many kinds of peptides [1-13] and proteins [14] such as collagen [1-3], elastin [4-7], resilin [7,8], silks [9,10] and others [11-14]. Synthetic methods [3,15-20] for such repetitive sequences may be useful tools and some perspective in clarifying the relationship between the sequences and their functions or the higher-order structures. The conventional methods for polypeptide synthesis have not always been suitable for preparing repetitive sequential polypeptides. The polymerization reactions (Fig. 1) from *N*-carboxy-amino acid anhydride (NCA) [21] have been useful for the preparation of homo-polypeptides and co-polypeptides but not useful for that of repetitive sequential polypeptides. Although solid-phase peptide synthesis [22,23] and polycondensation of active esters [3,19] of amino acids are available for the synthesis of sequential polypeptides, a large volume of solvents and a long reaction time are necessary during the synthetic processes. However, the thermal synthetic methods [24-31] require neither solvents nor a long reaction time for the polycondensation reaction.

In earlier studies [25], the thermal reactions of *N*-*t*-butyloxycarbonyl-amino acids (Boc-AA-OH) [32] for 1 or 2 h at temperatures were observed that were slightly higher than their melting points. The thermal reactions proceeded with the deprotection of Boc-group to give free amino acids, whereas using Boc-aspartic acid (Boc-Asp-OH) gave polypeptides (Fig. 2).

The results suggested that Boc-amino acids formed free amino groups possessing nucleophilic activity in the deprotected molecules. To incorporate this amino groups into polypeptide formation, we have tried the thermal reactions [25,29] of anhydrides of *N*-*t*-butyloxycarbonyl-aspartic acid (Boc-Asp-OH), *N*-*t*-butyloxycarbonyl-glutamic acid (Boc-Glu-OH) and *N*-*t*-butyloxycarbonyl-3-amino-glutaric acid (Boc-3-Agl-OH). The anhydride rather than free carboxy structure of the acidic amino acids might undergo a nucleophilic attack from the free amino group obtained by the decomposition of Boc group. During the thermal reactions, these compounds decomposed in the site of Boc-group to emit isobutene and carbon dioxide. The resulted acidic amino acid anhydrides except for 3-amino-glutaric acid anhydride each condensed to afford polypeptides [29] with molecular weights up to 12,400 Da.

The mixtures [26] composed of Boc-Asp anhydride and other *N*-Boc-amino acids were used for the thermal reactions to give polypeptide mixtures having molecular weight up to 2,000 Da. An intermediate aspartic anhydride structure derived

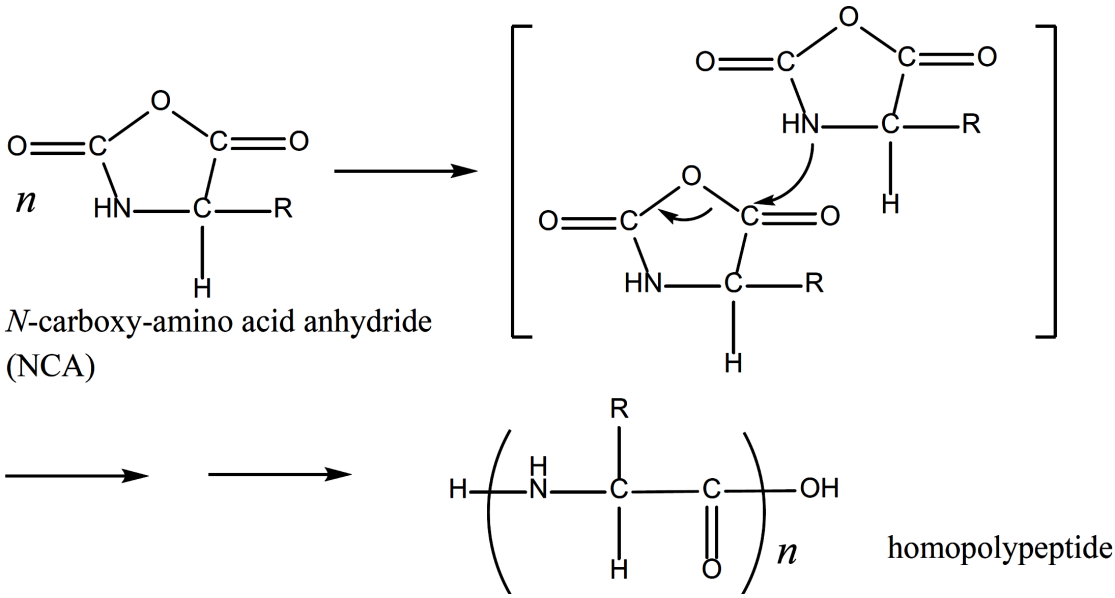

Fig. 1. Polypeptide formation from *N*-carboxy-amino acid anhydride

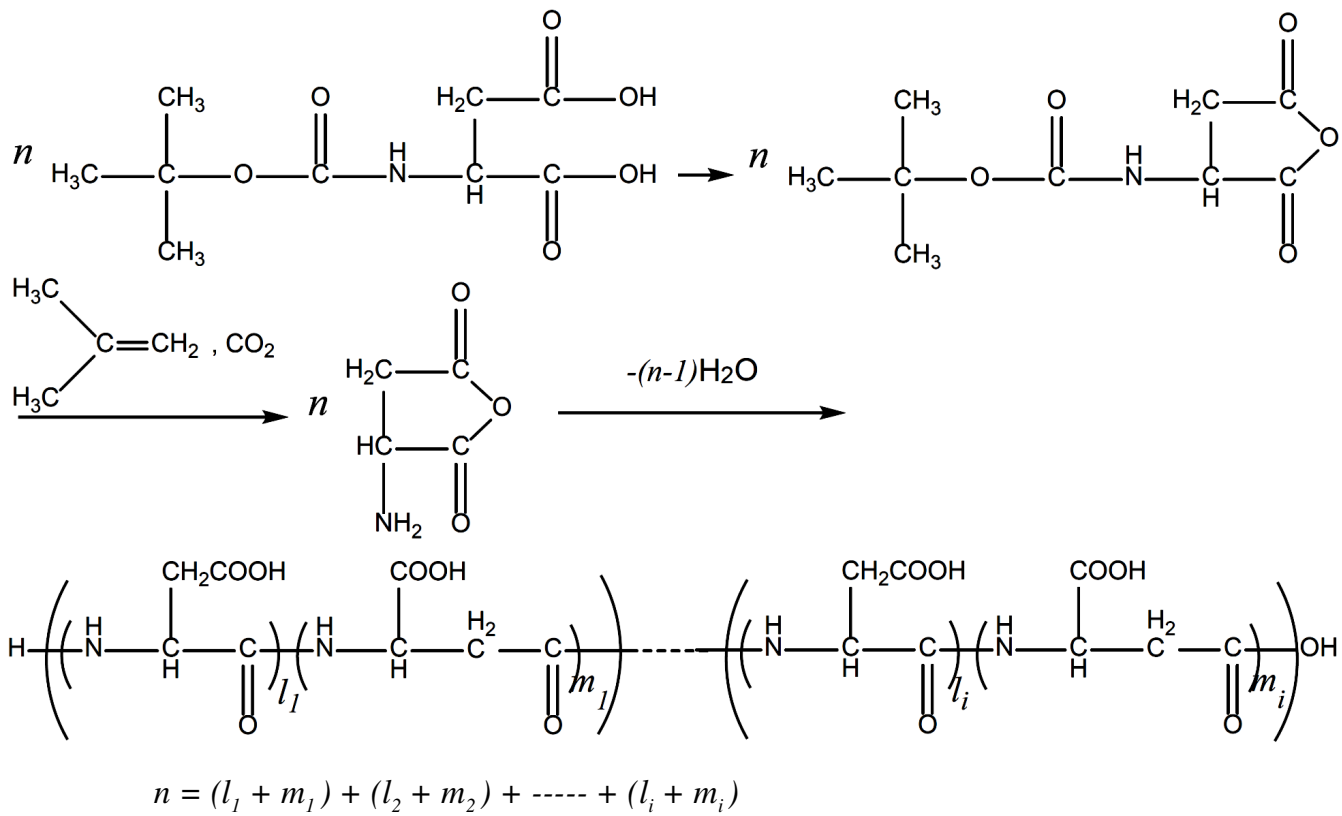

Fig. 2. Polypeptide formation from *N*-*t*-butyloxycarbonyl-aspartic acid upon thermal treatment

from the free acid structure has been suggested for facilitating the polymerization reactions. Based on these suggestions, *N*-Boc-dipeptides (Boc-Gly-Asp-OH, Boc-Ala-Asp-OH and Boc-Val-Asp-OH) and an *N*-Boc-tripeptide Boc-Gly-Gly-Asp-OH were used for thermal reactions [27,31] to afford sequential poly-peptides in a previous study (Fig. 3). The obtained polypeptide structures were determined as the sequential polypeptide with molecular weight up to 7,800 Da [31].

The higher molecular weight fractions after gel permeation chromatography were hydrolyzed to amino acids with the amino acid composition of the initial structure of Boc-peptides. However, such polypeptide structures with sequential Asp residues cannot always be found in the natural polypeptides. Whether the intermediate possessing anhydride structure at the *C*-terminal is necessary has not been determined for polymerization. The reaction activity of amide group (-C(=O)-NH-)

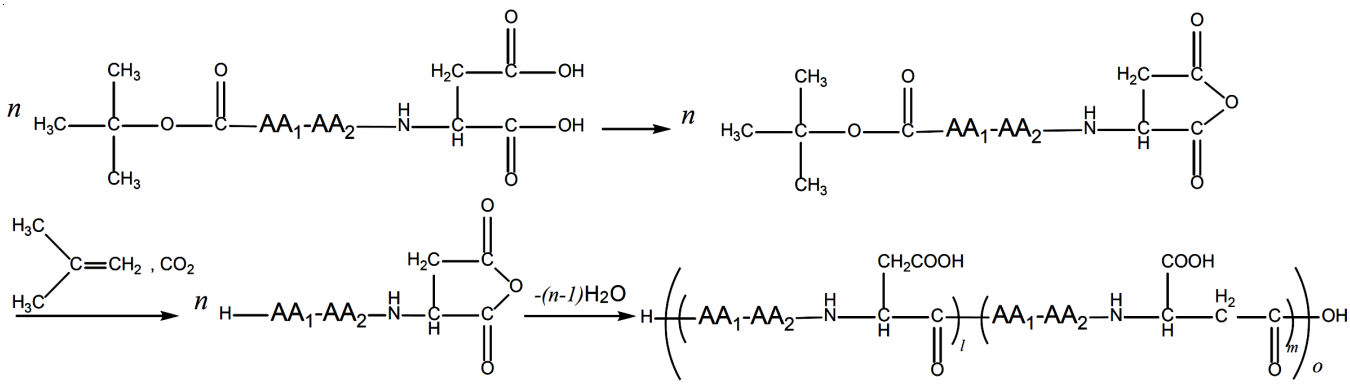

Fig. 3. Thermal reaction of Boc-AA1-AA2-Asp-OH (: Boc-Gly-Asp-OH; Boc-Ala-Asp-OH; Boc-Val-Asp-OH; Boc-Gly-Gly-Asp-OH)

of *N*-Boc-asparagine (Boc-Asn-OH) was used to give polypeptides up to 4,900 Da and the results were reported [30].

In the different studies, peptides derivatives having not active side chains like *N*-*t*- butyloxycarbonyl-prolyl-prolyl-glycine (Boc-Pro-Pro-Gly-OH) were heated to give polypeptides [28]. However, the detailed mechanisms and reaction conditions were not clarified. This article reports the thermal reactions of Boc-Pro-Pro-OH (**4**) and Boc-Pro- Gly-OH (**5**) which were prepared from Boc-Pro-OH (**1**) as shown in Fig. 4. In addition, polymerization reactions of *N*-Boc-tripeptides: Boc-Pro-Pro-Gly-OH (**7**), Boc- Pro-Pro-Gly-NH_2 (**8**) and Boc-Pro-Pro-Gly-OCH_3 (**9**) in Fig. 5 as well as Boc-Ala-Ala-Ala-OH (**17**) in Fig. 6 were performed. The products of the thermal reaction of sequential polypeptides like H-(Pro-Pro-Gly)$_n$-OH have a similar sequence to collagen [1-3]. These *N*-Boc-oligopeptides possess no polar reactive side chains. Boc-Ala-Ala-Ala-OH has a typically simple structure without reactive side chains, but might give water-soluble polypeptides H-(Ala-Ala-Ala)$_n$-OH under thermal treatment, whereas polypeptides H-(Gly-Gly-Gly)$_n$-OH has a much lower solubility than H-(Ala-Ala-Ala)$_n$-OH.

The six *N*-Boc-peptides *viz*. Boc-Pro-Pro-OH (**4**), Boc-Pro-Gly-OH (**5**), Boc-Pro-Pro- Gly-OH (**7**), Boc-Pro-Pro-Gly-NH_2 (**8**), Boc-Pro-Pro-Gly-OCH_3 (**9**) and Boc-Ala-Ala-Ala-OH (**17**) were separately heated without solvents at constant temperatures. During the thermal reactions, the weight change and the change in the heat formation were measured. The reaction mixtures were analyzed in relation to the molecular weight spread. From these results, the thermal reactions from *N*-Boc-tripeptides resulted in polypeptides. This study describes the detailed results and reveals a simple new thermal reaction for the polypeptide formation.

EXPERIMENTAL

A Jeol JLC-300 Amino Acid Analyzer was used for determining the amino acid composition in the hydrolysates of peptides (Jeol, Japan). A Hitachi 200-10 spectrophotometer was used for spectrophotometry measurements (Hitachi, Japan). A Jasco DIP-181 digital polarimeter (Jasco, Japan) was used for the measurement of the optical rotation of the peptide derivatives. A DT-40 thermal analyzer (Shimadzu, Japan) was used for thermal analysis. A Jasco Trimotor-V as the flow pump and a Jasco UVIDEC-100-IV spectrophotometer (Jasco, Japan) 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. A nuclear magnetic resonance (NMR) (Jeol FX-100 NMR system (Jeol, Japan)) was used for the collection of ^{1}H NMR spectra.

Standard polypeptides: Standard peptide compound H-(Pro-Pro-Gly)5-OH (*m.w.* = 1,274 Da) and H-(Pro-Pro-Gly)$_{10}$-OH (*m.w.* = 2,531 Da) were purchased from The Peptide Institute, Inc.

Preparation of peptide derivatives: Several *N*-*t*-butyloxycarbonyl-peptides (*N*-Boc-peptides) were prepared by the coupling of the corresponding *N*-Boc-amino acids and amino acids. The procedures are shown in Figs. 4 and 5.

Amino acid derivatives and other reagents: Proline (Pro-OH (**1**)), glycine (Gly-OH), alanine (Ala-OH) and di-*t*-butyl dicarbonate ((Boc)$_2$O) were purchased from Peptide Institute, Inc., Minoh-shi, Osaka, Japan. Glycinamide hydrochloride (HCl·Gly-NH_2) and glycine benzyl ester *p*-tosylate (Tos·Gly-OBzl) were purchased from Wako Pure Chemical Industries,

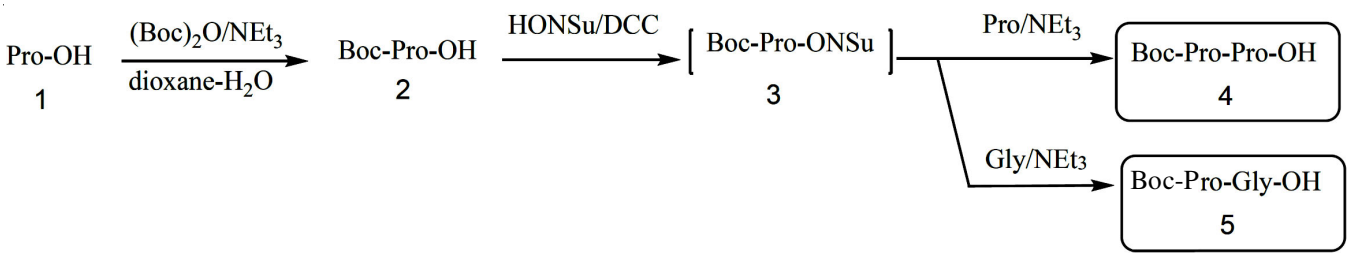

Fig. 4. Preparation procedure of Boc-Pro-Pro-OH (**4**) and Boc-Pro-Gly-OH (**5**) [Pro: L-proline, Boc-: *t*-butyloxycarbonyl-, HONSu: *N*-hydloxysuccinimide14, DCC: *N,N'*-dicyclohexylcarbodiimide, NEt_3: triethylamine; Gly: glycine]

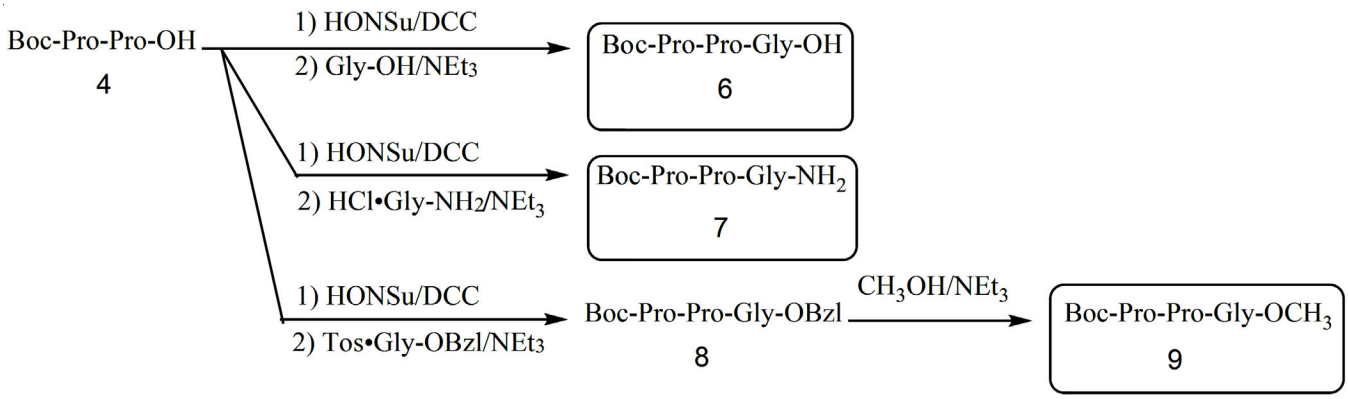

Fig. 5. Preparation procedure of Boc-Pro-Pro-Gly-X (**7**, **8**, and **9**) [Pro: L-proline, Boc-: *t*-butyloxycarbonyl-, HONSu: *N*-hydoxysuccinimide; DCC: dicyclohexylcarbodiimide, NEt_3: triethylamine, HCl·Gly-NH_2: glycinamide hydrochloride, Tos·Gly-OBzl: glycine benzyl ester *p*-tosylate]

Ltd [FUJIFILM Wako Pure Chemical Corporation since 2017), Osaka, Japan]. *N*-Hydroxysuccinimide (HONSu), *N,N′*-dicyclohexylcarbodiimide (DCC) and 4 M HCl in dioxane were procured from Watanabe Chemical Industries, Ltd. Hiroshima, Japan.

Boc-Pro-OH (2) and Boc-Pro-ONSu (3): Boc-Pro-OH (**2**) was prepared by protection of Pro (**1**) with $((Boc)_2O$ in a dioxane-water mixture. From Boc-Pro-OH (**2**) (10.8 g, 50 mmol) and HONSu [33] (5.75 g, 50 mmol) in presence of dicyclohexylcarbodiimide (DCC, 10.3 g, 50 mmol) in 50 mL ethyl acetate to obtain Boc-Pro-ONSu (**3**) (15.6 g, 100%) white crystal, which was recrystallized with 2-propanol (40 mL) to give white crystal (14.1 g, 90%, m.p.: 133-134 ºC (lit. [Ref. 33] 135-136 ºC).

Boc-Pro-Pro-OH (4): Boc-Pro-ONSu (**3**) (12.5 g, 40 mmol) and Pro-OH (**1**) (6.91 g, 60 mmol) in the presence of triethylamine (NEt_3, 60 mmol, 8.4 mL) in water 30 mL and dioxane 70 mL to Boc-Pro-Pro-OH (9.95 g, 80%), which was recrystallized with ethyl acetate (100 mL) to give white crystal (7.83 g, 63%, m.p.: 176-177 ºC). Elementary analysis: calcd. (found) % for $C_{15}H_{24}N_2O_5$: C, 57.68 (57.70); H, 7.74 (7.64); N, 8.97 (8.92). $[\alpha]_D^{27} = -119.0$ (*C* = 1.00, methanol).

Boc-Pro-Gly-OH (5): Boc-Pro-OH (**2**) (4.31 g, 20 mmol) was coupled with *N*-hydroxysuccinimide (HONSu, 2.77 g, 24 mmol) in the presence of DCC (2.53 g, 22 mmol) in 50 mL THF for 2 h at 0 ºC to give a suspension. After filtration of the suspension, the resulted filtrate solution of an active ester Boc-Pro-ONSu (**3**) was added in an aqueous solution including Gly-OH (2.25 g, 30 mmol) and triethylamine (6.3 mL, 45 mmol) at room temperature. After 2 days' reaction, the reaction solution after filtration was evaporated *in vacuo* and dissolved in ethyl acetate. The resulted ethyl acetate solution was combined with 10% $KHSO_4$ at pH 2 in a separatory funnel. The extraction with ethyl acetate was repeated two more times. The combined ethyl acetate solution was washed with 10% NaCl, dehydrated with $MgSO_4$ and evaporated to obtain a crude white solid. The crude product was recrystallized with ethyl acetate to give Boc-Pro-Gly-OH (**5**) (3.52 g, 65%). m.p.: 161-162 ºC. Elementary analysis: calcd. (found) % for $C_{12}H_{20}N_2O_5 \cdot 0.15NEt_3$: C, 53.77 (53.90); H, 7.90 (7.80); N, 10.46 (10.48). $[\alpha]_D^{23} = -67.3$ (*C* = 1.02, methanol).

Boc-Pro-Pro-Gly-OBzl (6): Boc-Pro-Pro-OH (**4**) (6.86 g, 22 mmol) was coupled with *N*-hydroxysucciimide (2.78 g, 24 mmol) in the presence of DCC (4.99 g, 24 mmol) to obtain Boc-Pro-Pro-ONSu (**3**) active ester, which was reacted with Tos·Gly-OBzl (7.41 g, 22 mmol). The resulted Boc-Pro-Pro-Gly-OBzl (**6**) was recrystallized with ethyl acetate and *n*-hexane to obtain 56%. m.p.: 109-111 ºC. Elementary analysis: calcd. (found) % for $C_{24}H_{33}N_3O_6$: C, 62.73 (62.58); H, 7.24 (7.21); N, 9.14 (9.12). $[\alpha]_D^{27} = -132$ (*C* = 1.02, methanol).

Boc-Pro-Pro-Gly-OH (7): Boc-Pro-Pro-Gly-OBzl (**6**) (4.60 g, 10 mmol) was hydrogenated in 30 mL ethanol in the presence of Pd-C for 45h. The reaction solution was filtered, and the resulting solution was evaporated and crystalized to give Boc-Pro- Pro-Gly-OH (**7**), 3.33 g (90%). m.p.: 72-74 ºC. Elementary analysis: calcd. (found) % for $C_{17}H_{27}N_3O_6 \cdot 0.4$ ethanol: C, 55.12 (54.82); H, 7.64 (7.61); N, 10.83 (11.06). $[\alpha]_D^{27} = -134.9$ (*C* = 1.02, methanol).

Boc-Pro-Pro-Gly-NH_2 (8): Boc-Pro-Pro-OH (**4**) (3.12 g, 10 mmol) was coupled with *N*-hydroxysucciimide (1.17 g, 11 mmol) in the presence of DCC (2.27 g, 11 mmol) to give Boc-Pro-Pro-ONSu active ester, which was reacted with HCl·Gly-NH_2 to result Boc-Pro-Pro-Gly-NH_2 (**8**) in the same reaction solution. From Boc-Pro-Pro-ONSu and HCl·Gly-NH_2 (1.22 g, 11 mmol) in the presence of NEt_3 in 60 mL CH_2Cl_2 and 30 mL DMF to give a white solid 3.91 g (87%). m.p.: 130 ºC. Elementary analysis: calcd. (found) % for $C_{17}H_{28}N_4O_5 \cdot H_2O$: C, 52.84 (53.23); H, 7.82 (7.22); N, 14.50 (14.40). $[\alpha]_D^{27} = -80.9$ (*C* = 1.00, methanol).

Boc-Pro-Pro-Gly-OCH_3 (9): Boc-Pro-Pro-Gly-OBzl (**6**) (4.58 g, 10 mmol) was mixed with CH_3OH (86 mL, 2.1 mol) in the presence of NEt_3 (14 mL, 100 mmol) and stirred for 25 h at room temperature. The reaction mixture was evaporated *in vacuo* to afford an oily product of Boc-Pro-Pro-Gly-OCH_3. The oily product was purified by a silica gel column chromatography using a mixture of ether-toluene-methanol (8:6:1 v/v). The main product fractions were collected and evaporated *in vacuo* to give a low melting point product (m.p.: 38-40 ºC); yield: 3.39 g (89%). Elementary analysis: calcd. (found) % for $C_{18}H_{29}N_3O_6 \cdot 2.5H_2O$: C, 55.73 (55.93); H, 7.66 (7.43); N, 10.83 (10.58). $[\alpha]_D^{25} = -134$ (*C* = 1.05, methanol).

Tos·Ala-OBzl (13): Ala (8.91 g, 100 mmol), benzyl alcohol (52.5 g, 485 mmol), *p*-toluene sulphonic acid (20.9 g, 110 mmol) and benzene (100 mL) were refluxed in a Dean-Stark apparatus for 20 h. The reaction mixture was evaporated to an

oily solution, which was then diluted with ether (100 mL). The resulted precipitate was filtered and recrystallized with ethanol-ether to give the product (27.1 g, 80%, m.p.: 112-113 ºC).

Boc-Ala-Ala-OBzl (14): The compounds related to Boc-Ala-Ala-Ala-OH were prepared as shown in Fig. 6. Ala-OH (**10**) was protected with *t*-butyloxycarbonyl group to give Boc-Ala-OH (**11**) (18.9g, 100 mmol), which was coupled with HONSu (1.27 g, 11 mmol) in the presence of DCC (24.8 g, 120 mmol) to give Boc-Ala-ONSu (**12**). Tos·Ala-OBzl (**13**) (alanine benzyl ester *p*-tosylate, 33.5 g, 110 mmol) was added to the reaction mixture to give Boc-Ala-Ala-OBzl (**14**) (yield: 30.8 g, 88%, m.p.: 64-65 ºC). Elementary analysis: calcd. (found) % for $C_{18}H_{26}N_2O_5$: C, 61.70 (62.00); H, 7.48 (7.63); N, 8.00 (8.07). $[\alpha]_D^{26}$ = –26.2 (*C* = 0.46, methanol).

HCl·Ala-Ala-OBzl (15): Boc-Ala-Ala-OBzl (**14**) (10.5 g, 30 mmol) was deprotected with 4 HCl dioxane to give HCl·Ala-Ala-OBzl (**15**) (yield: 7.74 g, 90%, m.p. 144-145 ºC). Elementary analysis: calcd. (found) % for $C_{13}H_{19}N_2O_3Cl{\cdot}0.1H_2O$: C, 54.11 (54.02); H, 6.71 (6.73); N, 9.71 (9.82). $[\alpha]_D^{25}$ = –32.7 (*C* = 1.01, methanol).

Boc-Ala-Ala-Ala-OBzl (16): Boc-Ala-ONSu (**12**) (5.73 g, 20 mmol) and HCl·Ala-Ala-OBzl **15** (5.74 g, 20 mmol) were reacted in the presence of NEt3 to afford Boc-Ala-Ala-Ala-OBzl (**16**) (yield: 8.43 g, 94%, 137-138 ºC). Elementary analysis: calcd. (found) % for $C_{21}H_{31}N_3O_6{\cdot}0.25H_2O$: C, 59.34 (59.54); H, 7.44 (7.57); N, 9.88 (9.61). $[\alpha]_D^{25}$ = –76.2 (*C* = 1.05, methanol).

Boc-Ala-Ala-Ala-OH (17): Boc-Ala-Ala-Ala-OBzl (**16**) (6.32 g, 15 mmol) was hydrogenated in the presence of 5% palladium on charcoal (Pd/C) in methanol to give Boc-Ala-Ala-Ala-OH (**17**). After filtration of the reaction mixture, the filtrate was evaporated *in vacuo* to give a crude product, which was recrystallized with tetrahydrofuran-*n*-hexane to afford a crystal product (4.28 g). Yield of compound **17** was 86% (m.p.: 184-185 ºC). $[\alpha]_D^{25}$ = –65.4 (*C* = 1.06, methanol). Elementary analysis: calcd. (found) % for $C_{14}H_{25}N_3O_6{\cdot}0.125THF$: C, 51.17 (50.87); H, 7.70 (7.82); N, 12.35 (12.22).

Thermal reactions and the weight recovery: *N*-Boc-Tripeptide derivatives (**7**, **8**, **9** and **17**) (0.50 mmol) were heated in each glass test tube (15 cm × 1.0 cm ϕ), which were set in an oil bath controlled at a constant temperature. The inside of the glass test tubes was under flowing nitrogen gas during the thermal reactions.

Gel permeation chromatography of thermal reaction mixtures: Each reaction mixture after thermal treatment in the glass tube was cooled to room temperature and dissolved in 5.0 M acetic acid (5 mL). The resulted solution was loaded onto a gel permeation chromatography (91 cm × 1.6ϕ cm, G-25F). Elution was carried out with 0.5 M acetic acid and the eluted solution was collected with a fraction collector. Each solution collected in the test tubes was monitored at 230 nm absorption.

RESULTS AND DISCUSSION

Thermal reactions of *N*-Boc-dipeptides: Depending on the angle from which structure of *N*-Boc-tripeptide substrate is viewed, they are composed of *N*-Boc-amino acids, *N*-Boc-dipeptides and amino acid segments. Because these segments may differently react according to their different structures, these compounds having similar segmental structure were heated separately. The amino acid derivatives, Boc-Ala-OH, Boc-Val-OH, Boc-Asp-OH were separately heated at 130-140 ºC to give free amino acid with the yield of more than 80% [25].

***N*-Boc-dipeptides:** Boc-Pro-Pro-OH (**4**) and Boc-Pro-Gly-OH (**5**) were heated in the same type but in different glass test tubes. After the thermal reactions of compound **4** (1.0 mmol) at 170 ºC for 4 h, the resulting product was recrystallized using THF and *n*-hexane to give crystalline solid (48%, m.p. 133-135 ºC). The ninhydrin reaction of the product was not detected. The ^{1}H NMR analysis of this compound in $CDCl_3$ showed as follows: δ = 4.10-4.20 (t, *J* = 8.1 Hz, 2H, two sites of -CH_2-C**H**-C(=O)-), 3.45-3.60 (t, *J* = 6.8 Hz, 4H, two sites of -N-C**H**$_2$-CH_2-),1.85-2.45 (m, 8H, two sites of -CH_2-C**H**$_2$-C**H**$_2$-CH-). The data showed that the product was diketopiperazine (cyclic dipeptide: Cyclo-(Pro-Pro)). The thermal reaction of compound **4** for 24 h at 170 ºC produced 38% decrease in mass, which corresponds the total of the complete Boc group deprotection and dehydration between two prolines to cyclo-(Pro-Pro).

The Boc-Pro-Gly-OH (**5**) (1.0 mmol) was heated at 150 ºC for 24 h to reveal 43% decrease in mass, which corresponds the complete Boc-group deprotection and dehydration between a proline molecule and glycine molecule to cyclo-(Pro-Gly). The results that the thermal reactions of *N*-Boc-dipeptides afforded cyclic dipeptides were not clearly observed in the thermal reactions of Boc-Gly-Asp [31]. The compound having a reactive side chain at *C*-terminal like Boc-Gly-Asp can make β-peptide bond after melting and deprotection of Boc-group. However, *N*-Boc-dipeptides having no reactive side chains may cyclize to diketopiperazines. Therefore, these results suggested that longer *N*-Boc-peptides like *N*-Boc-tripeptides are necessary to reveal polypeptides for thermal reaction.

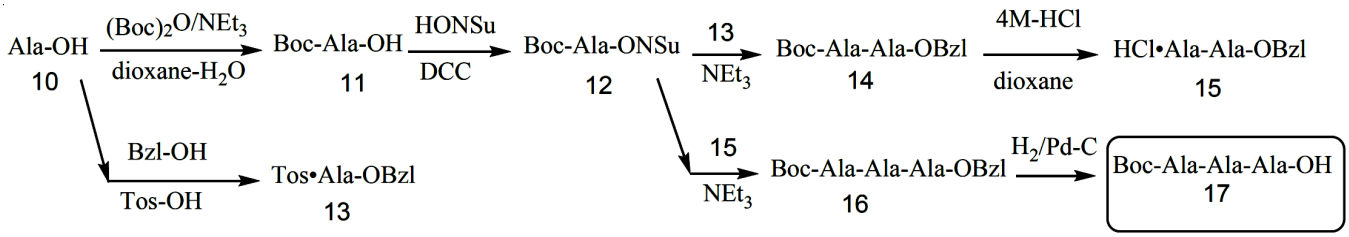

Fig. 6. Preparation procedure of Boc-Ala-Ala-Ala-OH (**17**) [Ala: L-alanine, Boc-: *t*-butyloxycarbonyl-, HONSu: *N*-hydoxysuccinimide, DCC: dicyclohexylcarbodiimide, NEt_3: triethylamine, Tos·Ala-OBzl (**13**): alanine benzyl ester *p*-tosylate, 4 M HCl: 4 mol/L HCl in dioxane, H_2/Pd-C: hydrogenation over palladium on charcoal]

Thermal analysis of Boc-Pro-Pro-Gly-X (7, 8 and 9) derivatives: The peptide derivatives Boc-Pro-Pro-Gly-OH (**7**) as well as Boc-Pro-Pro-Gly-NH_2 (**8**) were chosen as the substrates for the thermal syntheses. A small amount of sample was heated in a thermal analyzer (Shimadzu DT-40) under helium stream to monitor the thermal gravimetry (TG) and different thermal analysis (DTA). Figs. 7 and 8 show the thermal analysis data (TG and DTA) from compounds **7** and **8**, respectively. However, the thermal analysis of the derivative Boc-Pro-Pro-Gly-OCH_3 (**9**) was not caried out, because of the much lower melting point and the difficulty of sample making for the thermal analysis.

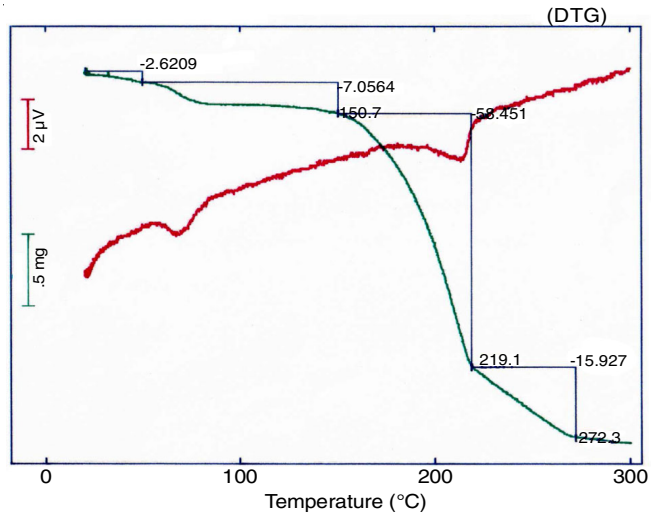

Fig. 7. TG (thermal gravimetry, green line) and DTA (differential thermal analysis, red line) of Boc-Pro-Pro-Gly-OH (**7**)

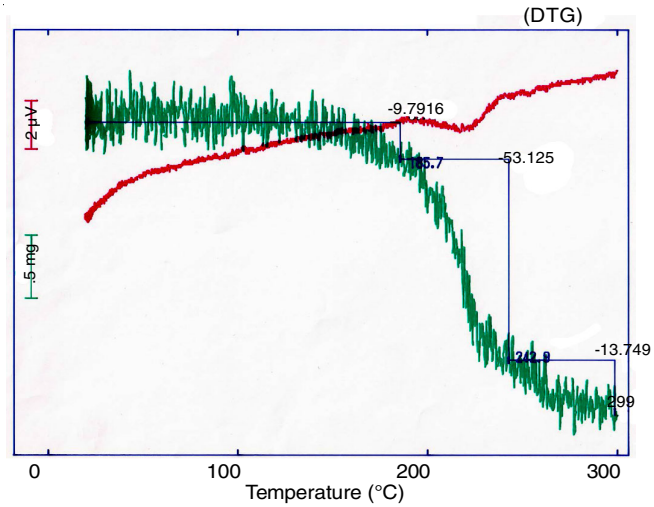

Fig. 8. TG (thermal gravimetry, green line) and DTA (differential thermal analysis, red line) of Boc-Pro-Pro-Gly-NH_2·H_2O (**8**)

Boc-Pro-Pro-Gly-OH (**7**) (3.10 mg) was heated from 50 to 300 ºC at a rate of 5 ºC/min. The first endothermic peak between 50 and 100 ºC corresponds to the melting point of Boc-Pro-Pro-Gly-OH (72-74 ºC). The second endothermic peak corresponds to the decomposition of Boc-group and the following dehydration between Pro-Pro-Gly-OH molecules to give polypeptides. At a little lower temperature (150 to 160 ºC) before the second endothermic peak, the decrease in weight started. The results suggested that the decomposition of compound **7** started at 150 to 160 ºC. Such degrees will be proper for polymerization of Boc-Pro-Pro-Gly-OH (**7**).

Boc-Pro-Pro-Gly-NH_2·H_2O (**8**) (3.10 mg) was also heated from 50 to 300 ºC at a rate of 5 ºC/min. A small endothermic peak at a little higher temperature than 100 ºC is according to the melting point of Boc-Pro-Pro-Gly-NH_2·H_2O (109-111 ºC). The second endothermic peak corresponds to the decomposition of Boc-group and the following dehydration between H-Pro-Pro-Gly-NH_2 to give polypeptides. At a little lower temperature (150 to 160 ºC) before the second endothermic peak, the decrease in weight of Boc-Pro-Pro-Gly-NH_2·H_2O was also started, which suggests the thermal reactions.

Mass spectrometry of the gasses from compounds 7 and 8 upon thermal: Figs. 9 and 10 show the MS data emitted gases during the thermal analysis of compounds **7** and **8**, respectively. The gas mixture generated during the thermal reaction was directly introduced to a mass spectrometry (Shimadzu QP-1000A). Water (m/z = 18), carbon dioxide (m/z = 44), and isobutene (m/z = 56) were detected by selective ion monitoring. In the thermal reaction of Boc-Pro-Pro-Gly-OH (**7**), ammonia was not detected at higher temperature, although in the reaction of Boc-Pro-Pro-Gly-NH_2 H_2O (**8**), ammonia (m/z = 17) was detected but water was not detected. Water molecule of the compound **7** is a kind of crystal water, which is estimated to have been evaporated at a lower temperature. The generated gases during the thermal analysis suggested that deprotection and condensation proceeded in the thermal reaction.

Thermal reactions of compounds 7, 8, 9 and 17: *N*-Boc-Pro-Pro-Gly-X derivatives (**7**, **8** and **9**, 0.50 mmol) were heated for a constant reaction time at a constant temperature. The weight recovery of substrate **7** during the thermal at 130, 140 and 150 ºC was plotted against the reaction time as shown in Fig. 11. After 16-24 h reaction, the rate of weight decrease became slow. The weight loss after 24 h reaction at 150 ºC was larger than the decrease (32% loss: 68% recovery) equivalent to the total deprotection and the condensation between peptides. This observation might be explained by substrate sublimation.

The weight recovery of substrates **8**, **9** and **17** during the thermal is shown in Figs. 12-14, respectively. The thermal reactions of Boc-Pro-Pro-Gly-OH (**7**) at 140 ºC, Boc-Pro-Pro-Gly-NH_2 (**8**) at 160 ºC, Boc-Pro-Pro-Gly-OH (**9**) at 160 ºC Boc-Ala-Ala-Ala-OH (**17**) at 170 ºC were estimated to be complete in 1 or 2 days. However, the recovery data may not reflect sublimation from the reaction mixture might happen in the thermal reactions. This research contributed to analyzing the remaining products after the thermal reactions, whereas sublimation of the peptide derivatives will be discussed in other work. The molecular weight feature of the remaining products was analyzed by gel permeation chromatography.

Gel permeation chromatography of compounds 7, 8, 9 and 17: The reaction mixture after thermal reaction at 150 ºC was dissolved in 0.5 M acetic acid and the solution was loaded onto a Sephadex G-25 column. The gel permeation chromatogram (GPC) of the reaction mixture from Boc-Pro-Pro-Gly-OH (**7**) is shown in Fig. 15.

With an increase in the reaction time, the higher molecular weight fractions moved to the further higher molecular weight

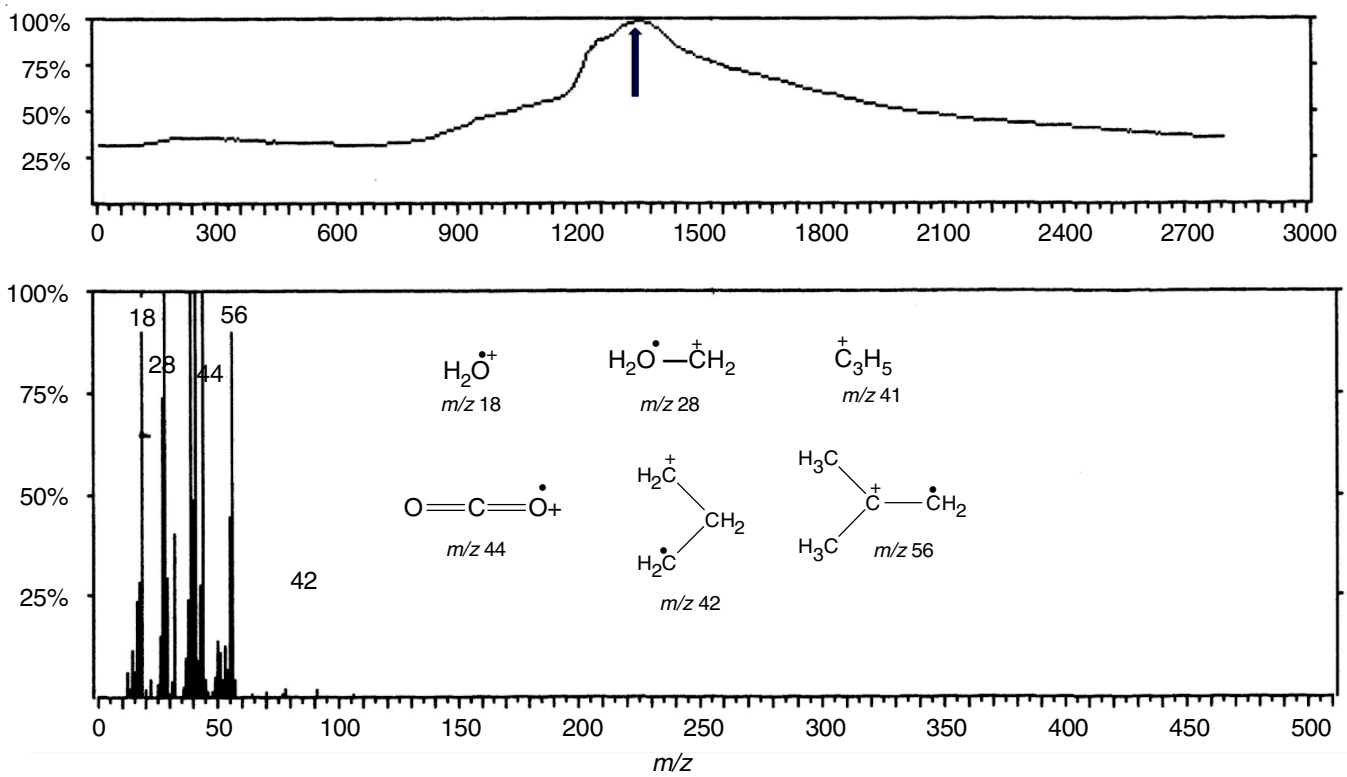

Fig. 9. Mass spectrometry of the gases emitted during the thermal reaction of Boc-Pro-Pro-Gly-OH (**7**) in the thermal analysis

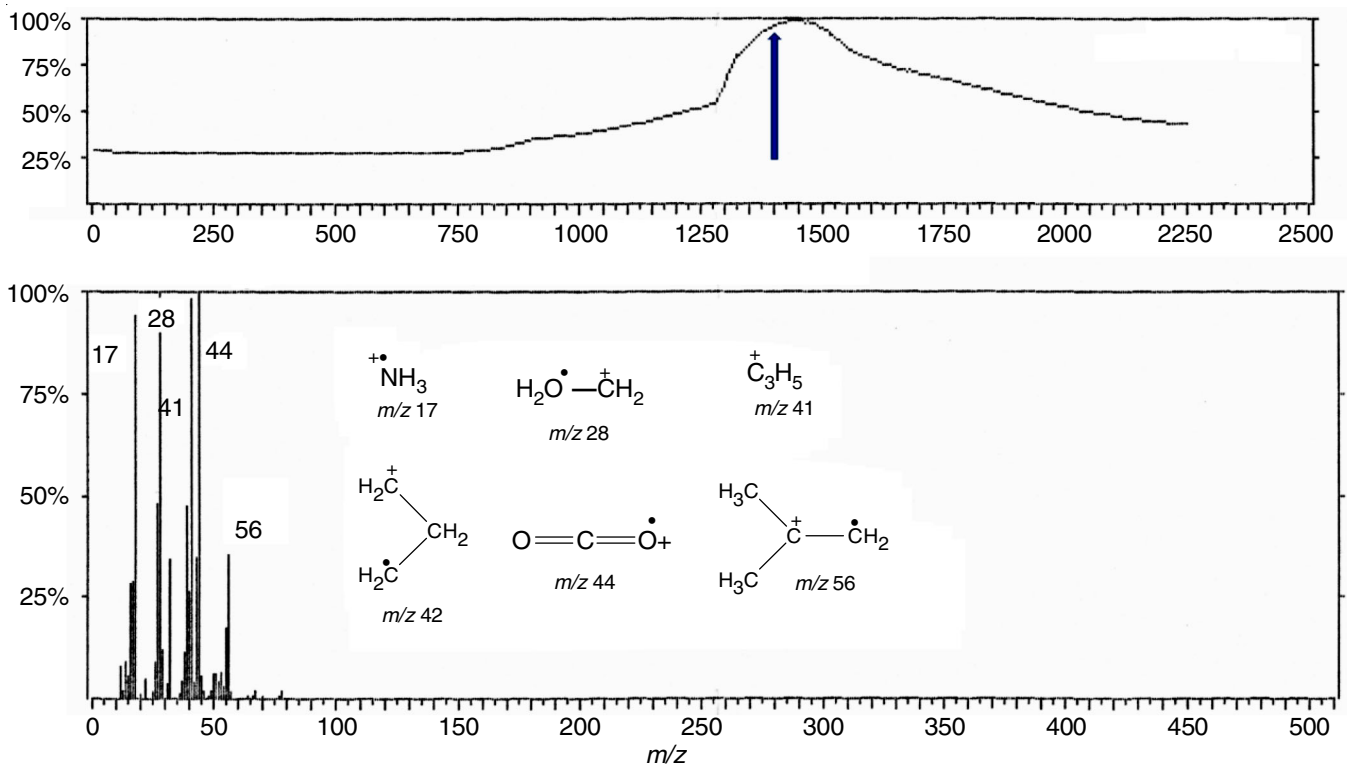

Fig. 10. Mass spectrometry of the gases emitted during the thermal reaction of Boc-Pro-Pro-Gly-NH_2 (**8**) in the thermal analysis

fraction area. Comparing the chromatograms with those of standards: (a) H-(Pro-Pro-Gly)$_{10}$-OH (*m.w.* = 2,531) and (b) H-(Pro-Pro-Gly)$_5$-OH (*m.w.* = 1,274), the highest molecular fractions of the chromatogram of the reaction mixture for 16 h was in the range between standards (a) and (b).

The higher molecular-weight fractions than H-(Pro-Pro-Gly)$_5$-OH (*m.w.* = 1,274) were combined and lyophilized after GPC to give amorphous powder. The yield was 5.3 mg from the reaction at 150 ºC for 16 h. The other reaction mixtures were purified in the same manner to give higher molecular

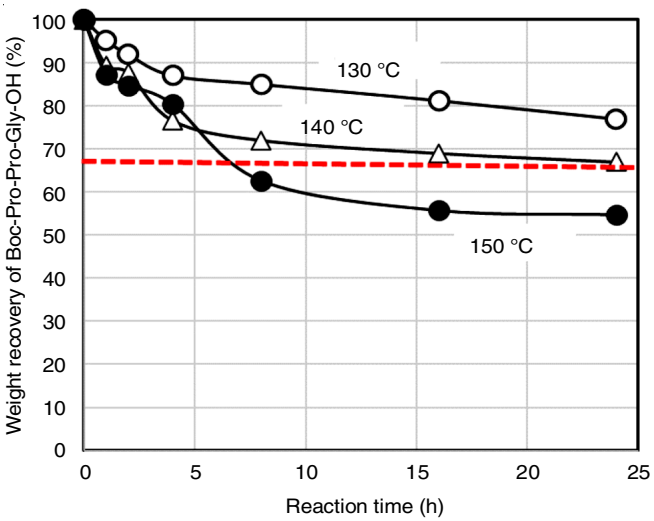

Fig. 11. Weight recovery of Boc-Pro-Pro-Gly-OH (**7**) during the thermal reaction [the dashed line indicates the estimated level after complete deprotection and peptide formation]

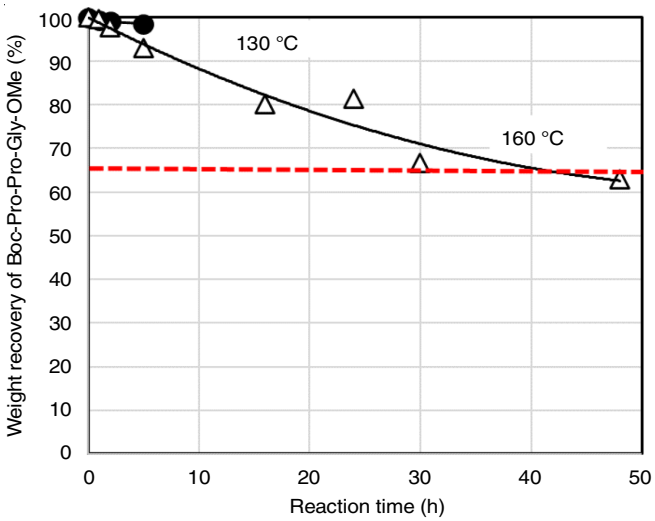

Fig. 13. Weight recovery of Boc-Pro-Pro-Gly-OCH_3 (**9**) during the thermal reaction [the dashed line indicates the level after complete deprotection and peptide formation]

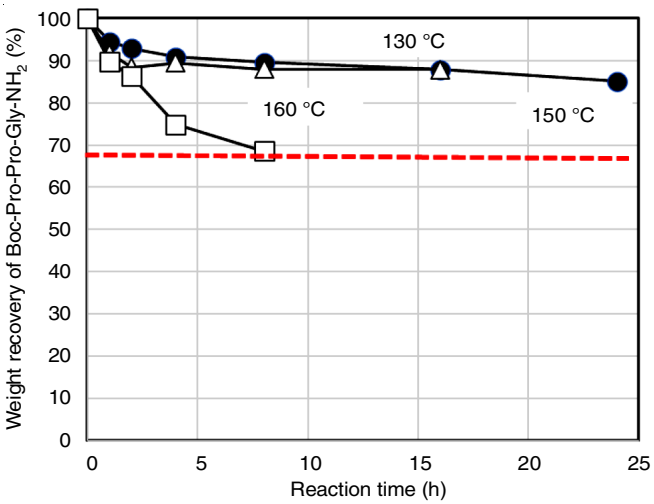

Fig. 12. Weight recovery of Boc-Pro-Pro-Gly-NH_2 (**8**) during the thermal reaction [the dashed line indicates the level after complete deprotection and peptide formation]

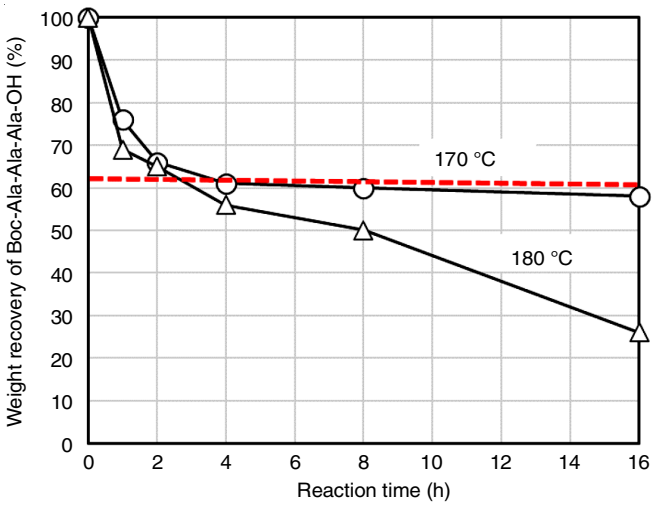

Fig. 14. Weight recovery during the thermal reaction of Boc-Ala-Ala-Ala-OH (**17**) [the dashed line indicates the level after complete deprotection and peptide formation]

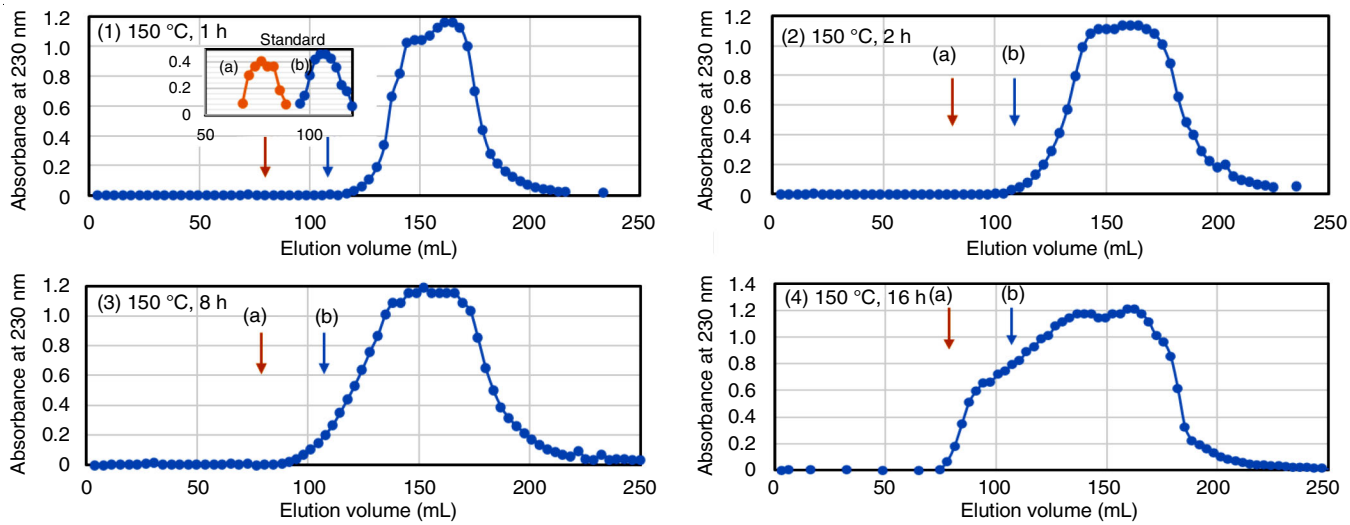

Fig. 15. Gel permeation chromatography of the thermal reaction mixtures from Boc-Pro-Pro-Gly-OH (**7**) at 150 ºC.; **(a)** H-(Pro-Pro-Gly)$_{10}$-OH, (*m.w.* = 2,531); **(b)** H-(Pro-Pro-Gly)$_5$-OH, (*m.w.* = 1,274)

weight (1-5 mg). The average molecular weight of the purified product was estimated at the range of 1,200-2500 Da by HPLC analysis using gel permeation chromatography (TSK-GEL 3000PW). The IR spectrum of the product appeared similarly to that of the standard H-(Pro-Pro-Gly)$_5$-OH. The higher molecular weight fractions were hydrolyzed in 6 M HCl and the amino acids in the hydrolysate were analyzed. The molar ratio of Pro and Gly in the hydrolysate of the higher molecular weight fraction was almost 2:1. The results of the above mentioned data indicate that the higher molecular weight product would be a sequential polypeptide like H-(Pro-Pro-Gly)$_n$-OH.

The GPCs from Boc-Pro-Pro-Gly-NH$_2$ (**8**) at 160 ºC, Boc-Pro-Pro-Gly-OCH$_3$ (**9**) at 160 ºC, Boc-Ala-Ala-Ala-OH (**17**) at 170 ºC and Boc-Ala-Ala-Ala-OH (**17**) at 180 ºC are shown in Figs. 16-19, respectively. The GPCs of the reaction mixtures of compounds **8**, **9** and **17** showed the similar pattern to the chromatogram of the reaction mixture of compound **7**. The higher molecular weight fractions increased but the lower molecular weight fractions decreased with the reaction time. Although these compounds have different leaving groups (-OH for **7** and **17**; -NH$_2$ for **8**; -OCH$_3$ for **9**), the reaction rate seems to be at a similar level.

The higher molecular-weight fractions than H-(Pro-Pro-Gly)5-OH (*m.w.* = 1,274) were combined and lyophilized after GPCs from the reactions to give amorphous powder products. The yield in several cases were 0.7 mg (from **8**, 160 ºC, 8 h), 0.8 mg (from **9**, 160 ºC, 30 h), 0.5 mg (from **17**, 170 ºC, 16 h), 8.1 mg (from **17**, 180 ºC, 8 h) and 15.5 mg (from **17**, 180 ºC, 16 h).Comparing the yields above cited with that 5.3 mg from Boc-Pro-Pro-Gly-OH (**7**) at 150 ºC for 16 h, the compounds **7**

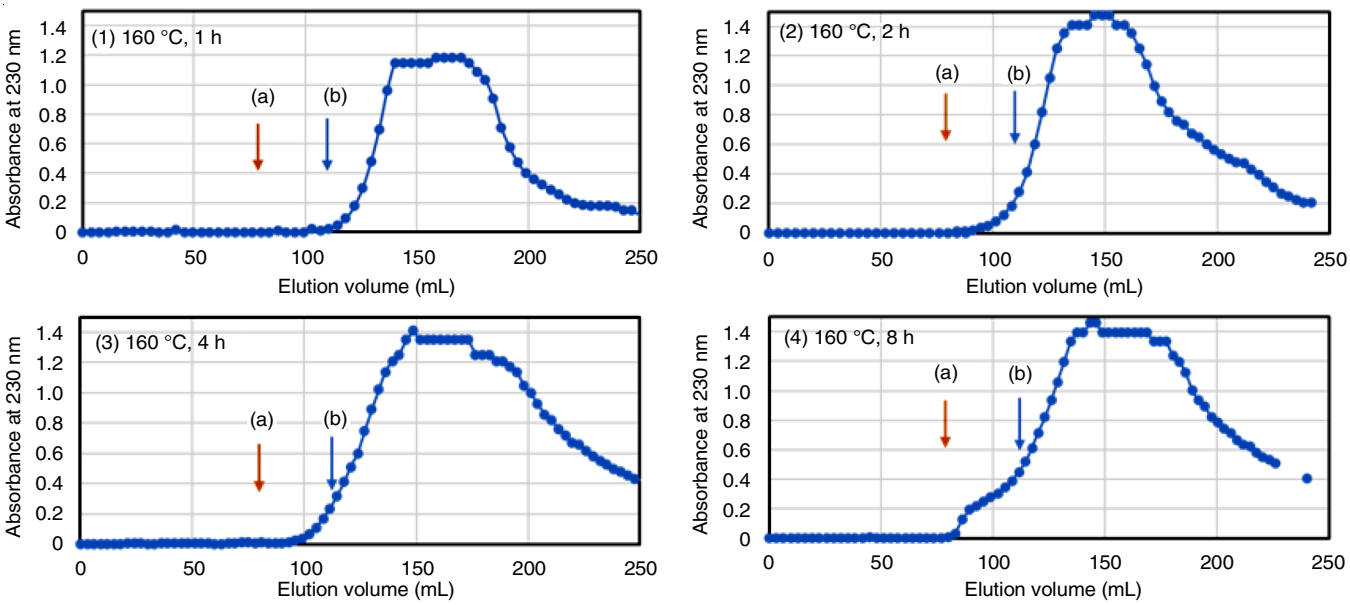

Fig. 16. Gel permeation chromatograms of the thermal reaction materials from Boc-Pro-Pro-Gly-NH$_2$ (**8**) at 160 ºC. (a) H-(Pro-Pro-Gly)$_{10}$-OH (*m.w.* = 2,531) and (b) H-(Pro-Pro-Gly)$_5$-OH (*m.w.* = 1,274)

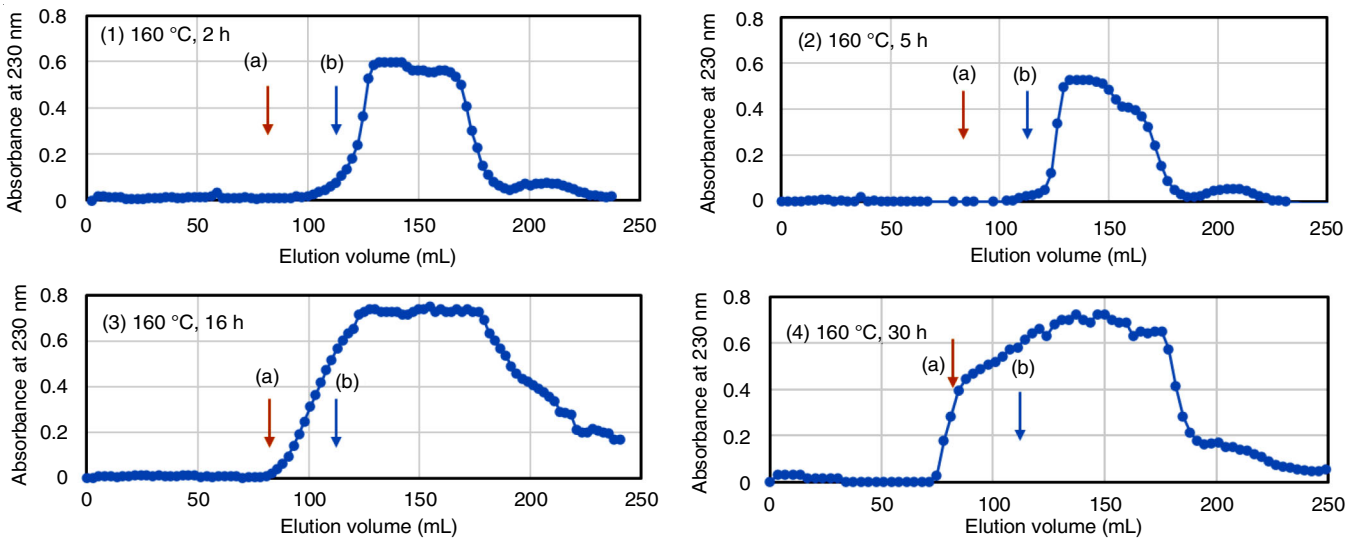

Fig. 17. Gel permeation chromatograms of the thermal reaction materials from Boc-Pro-Pro-Gly-OCH$_3$ (**9**) at 160 ºC. (a) H-(Pro-Pro-Gly)$_{10}$-OH (*m.w.* = 2,531) and (b) H-(Pro-Pro-Gly)$_5$-OH (*m.w.* = 1,274)

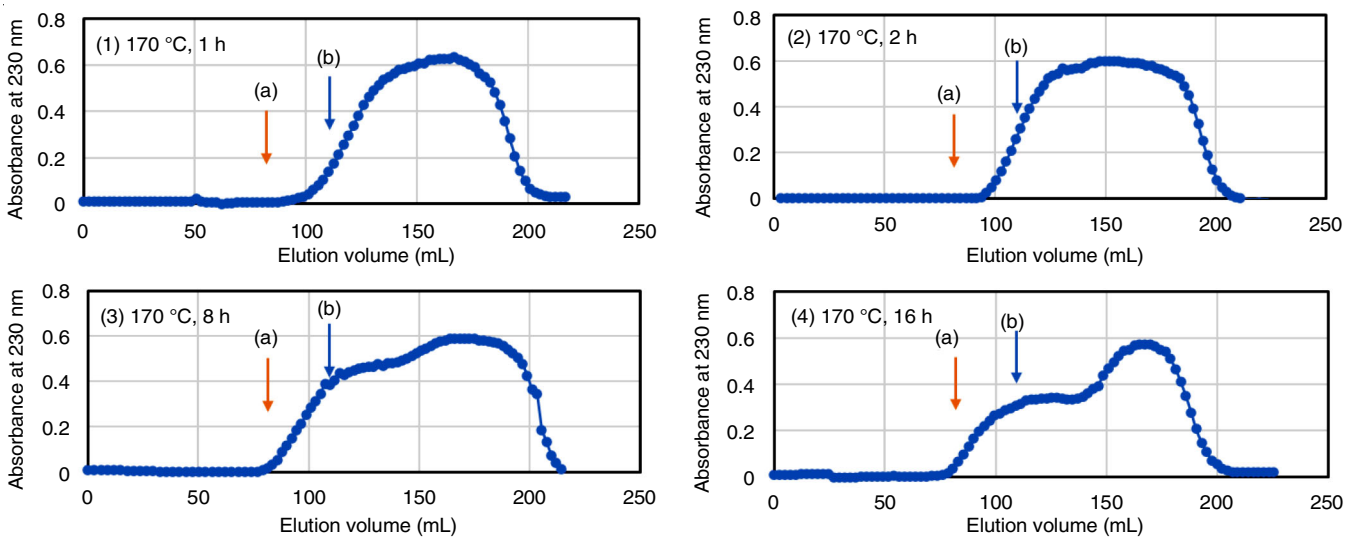

Fig. 18. Gel permeation chromatograms of the thermal reaction materials from Boc-Ala-Ala-Ala-OH (**17**) at 170 ºC. (a) H-(Pro-Pro-Gly)$_{10}$-OH (*m.w.* = 2,531) and (b) H-(Pro-Pro-Gly)$_5$-OH (*m.w.* = 1,274)

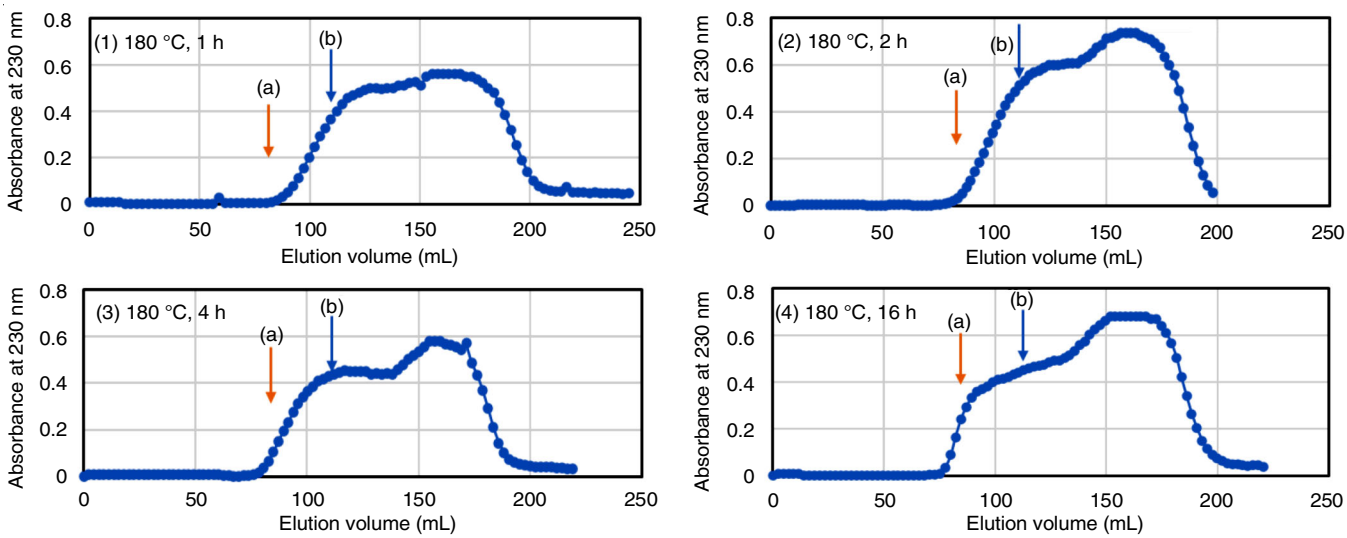

Fig. 19. Gel permeation chromatograms of the thermal reaction materials from Boc-Ala-Ala-Ala-OH (**17**) at 180 ºC. (a) H-(Pro-Pro-Gly)$_{10}$-OH (*m.w.* = 2,531) and (b) H-(Pro-Pro-Gly)$_5$-OH (*m.w.* = 1,274)

and **17** having *C*- terminal -OH gave higher yields than other leaving groups (-NH_2 (**8**) and -OCH_3 (**9**)).

Although peptide bond formation proceeded according to the kinds of leaving group, the reaction procedure may consist of three steps. The first step is assumed to be the melting of the compounds, whereas the second step would be the deprotection of the Boc-group, which emits carbon dioxide and isobutene to afford free amino group. The deprotection reaction upon thermal is confirmed in the measurement with mass spectrometry measurement (Figs. 9 and 10). The third step would be peptide formation by the attack of a free amino group to a carboxy group of the different molecules. The third step results in emitting H_2O (from **7**), NH_3 (from **8**) and CH_3OH (from **9**) to give polypeptides. The reaction mechanism is explained in Fig. 20.

The thermal reaction of Boc-Ala-Ala-Ala-OH (**17**) is also estimated to proceed with a similar mechanism to that in Fig. 21 as for the reaction of Boc-Pro-Pro-Gly-X (X, -OH (**7**); X: -NH_2 (**8**); X: OCH_3 (**9**)).

Conclusions

Based on the results and discussion, it is concluded the reactivity of *N*-Boc-amino acids, *N*-Boc-dipeptides and *N*-Boc-tripeptides in the thermal reactions without solvents as follows:

1. *N*-Boc-amino acids (Boc-Ala-OH, Boc-Val-OH, Boc-Pro-OH, *etc.*) without reactive side chains change to free α-amino acids in the thermal reactions for several hours at the temperatures near melting points.

2. *N*-Boc-dipeptides (Boc-Pro-Pro-OH, Boc-Pro-Gly-OH) without reactive side chains change to cyclic dipeptides (diketopiperazines: cyclo-(Pro-Pro), cyclo-(Pro-Gly)) in the thermal reactions for several hours at the temperatures near melting points.

3. *N*-Boc-tripeptides: Boc-Pro-Pro-Gly-OH (**7**), Boc-Pro-Pro-Gly-NH_2 (**8**), Boc-Pro-Pro-Gly-OCH_3 (**9**) and Boc-Ala-

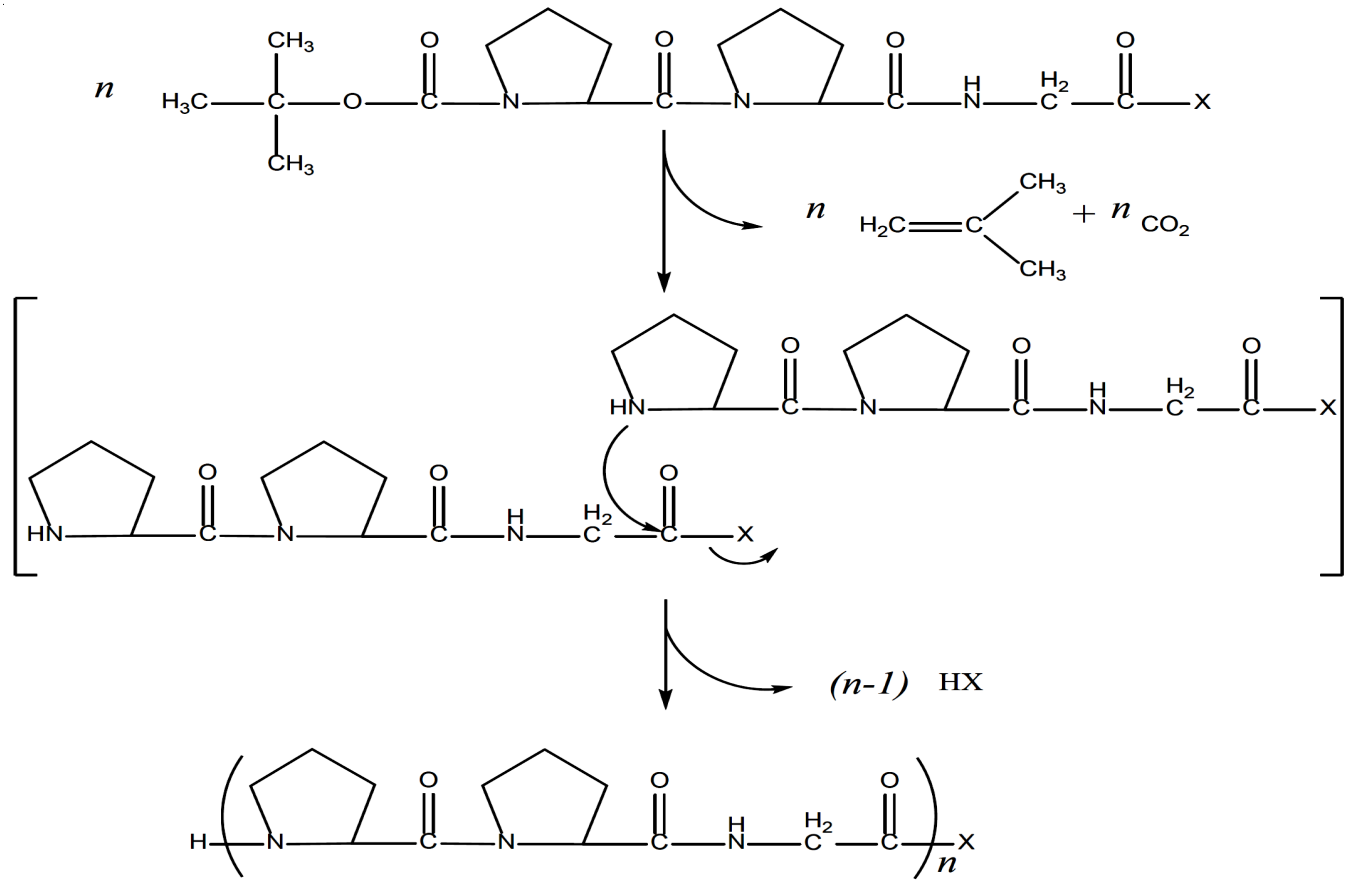

Fig. 20. A proposed reaction mechanism of thermal polymerization of Boc-Pro-Pro-Gly-X (X: -OH (**7**), -NH_2 (**8**), -OCH_3 (**9**))

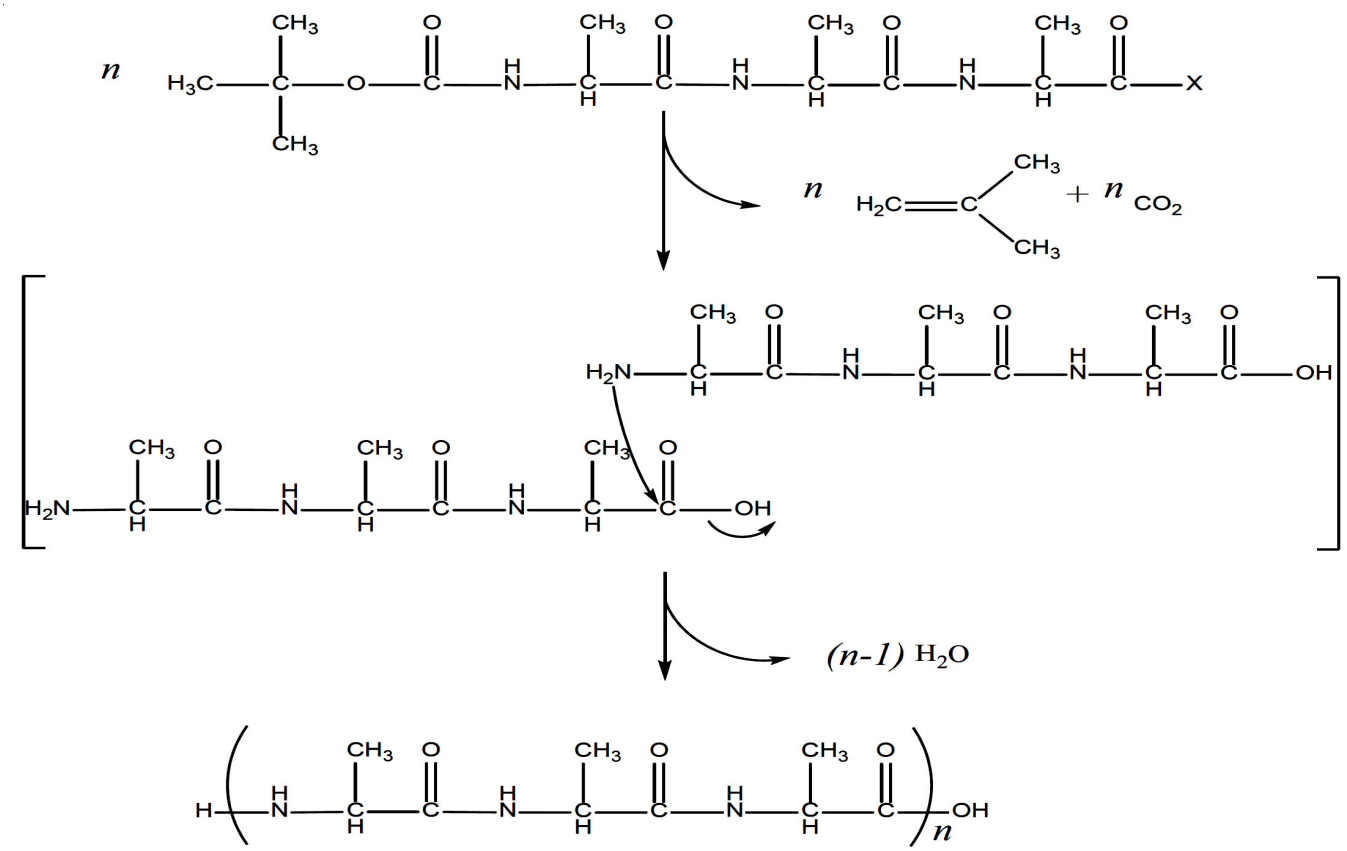

Fig. 21. A proposed reaction mechanism of thermal polymerization of Boc-Ala-Ala-Ala-OH (**17**)

Ala-Ala-OH (**17**) produce repetitive polypeptides in the thermal reactions for several hours at the temperatures near melting points.

4. The highest molecular masses of polypeptides obtained from *N*-Boc-tripeptides: compounds **7**, **8**, **9** and **17** were in the range of about 1,272 to 2,531 g/mol.

5. If the thermal reactions of *N*-Boc-tripeptides: compounds **7**, **8**, **9** and **17** were carried out at a higher temperature for a long time, the mass of *N*-Boc-tripeptides decreased much more than expected for the total decrease due to deprotection and dehydration. Sublimation from the reaction mixtures may be necessary for future work.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. J. Greenberg, L. Fishman and M. Levy, *Biochemistry*, **3**, 1826 (1964); https://doi.org/10.1021/bi00900a005
2. R. Sakai, S. Ikeda and T. Isemura, *Bull. Chem. Soc. Jpn.*, **40**, 2890 (1967); https://doi.org/10.1246/bcsj.40.2890
3. S. Sakakibara, Y. Kishida, Y. Kikuchi, R. Sakai and K. Kakiuchi, *Bull. Chem. Soc. Jpn.*, **41**, 1273 (1968); https://doi.org/10.1246/bcsj.41.1273
4. W.R. Gray, L.B. Sandberg and J.A. Foster, *Nature*, **246**, 461 (1973); https://doi.org/10.1038/246461a0
5. S. Sumiyoshi, K. Suyama, N. Tanaka, T. Andoh, K. Tomohara, A. Nagata, S. Taniguchi, I. Maeda and T. Nose, *Sci. Rep.*, **12**, 19414 (2022); https://doi.org/10.1038/s41598-022-23940-0
6. J. Ozsvar, C. Yang, S.A. Cain, C. Baldock, A. Tarakanova and A.S. Weiss, *Front. Bioeng. Biotechnol.*, **9**, 643110 (2021); https://doi.org/10.3389/fbioe.2021.643110
7. Z. Chen, Q. Zhang, H. Li, Q. Wei, X. Zhao and F. Chen, *Bioact. Mater.*, **6**, 589 (2021); https://doi.org/10.1016/j.bioactmat.2020.09.003
8. R.E. Lyons, D.C.C. Wong, M. Kim, N. Lekieffre, M.G. Huson, T. Vuocolo, D.J. Merritt, K.M. Nairn, D.M. Dudek, M.L. Colgrave and C.M. Elvin, *Insect Biochem. Mol. Biol.*, **41**, 881 (2011); https://doi.org/10.1016/j.ibmb.2011.08.002
9. O. Hakimi, D.P. Knight, F. Vollrath and P. Vadgama, *Composites B Eng.*, **38**, 324 (2007); https://doi.org/10.1016/j.compositesb.2006.06.012
10. D.J. Perry, D. Bittencourt, J. Siltberg-Liberles, E.L. Rech and R.V. Lewis, *Biomacromolecules*, **11**, 3000 (2010); https://doi.org/10.1021/bm1007585
11. S. Clements, H. Mehansho and D.M. Carlson, *J. Biol. Chem.*, **260**, 13471 (1985); https://doi.org/10.1016/S0021-9258(17)38745-8
12. K. Shiba and T. Minamisawa, *Biomacromolecules*, **8**, 2659 (2007); https://doi.org/10.1021/bm700652b
13. D. Jiang, C. Yao, N. Hu and Y. Liu, *Molecules*, **26**, 6207 (2021); https://doi.org/10.3390/molecules26206207
14. H. Zhu, E. Sepulveda, M.D. Hartmann, M. Kogenaru, A. Ursinus, E. Sulz, R. Albrecht, M. Coles, J. Martin and A.N. Lupas, *eLife*, **5**, 16761 (2016); https://doi.org/10.7554/eLife.16761
15. R.S. Rapaka, K. Okamoto and D.W. Urry, *Int. J. Pept. Protein Res.*, **11**, 109 (1978); https://doi.org/10.1111/j.1399-3011.1978.tb02830.x
16. R.S. Rapaka and D.W. Urry, *Int. J. Pept. Protein Res.*, **11**, 97 (1978); https://doi.org/10.1111/j.1399-3011.1978.tb02829.x
17. J.H. Johns, ed. by B. Weinstein, Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins, Marcel Dekker Inc., New York, vol. 4, pp. 23-63 (1977).
18. R.S. Farmer and K.L. Kiick, *Biomacromolecules*, **6**, 1531 (2005); https://doi.org/10.1021/bm049216+
19. I.F. Eggen, F.T. Bakelaar, A. Petersen, P.B.W. Ten Kortenaar, N.H.S. Ankone, H.E.J.M. Bijsterveld, G.H.L. Bours, F.E.L. Bellaj, M.J. Hartsuiker, G.J. Kuiper and E.J.M. Ter Voert, *J. Pept. Sci.*, **11**, 633 (2005); https://doi.org/10.1002/psc.670
20. B. Lewandowski, G. De Bo, J.W. Ward, M. Papmeyer, S. Kuschel, M.J. Aldegunde, P.M.E. Gramlich, D. Heckmann, S.M. Goldup, D.M. D'Souza, A.E. Fernandes and D.A. Leigh, *Science*, **339**, 189 (2013); https://doi.org/10.1126/science.1229753
21. Y. Imanishi, *Pure Appl. Chem.*, **53**, 715 (1981); https://doi.org/10.1351/pac198153030715
22. R. Nyfeler, Peptide Synthesis *via* Fragment Condensation, Peptide Synthesis Protocols, In: Methods in Molecular Biology, Springer Nature, vol. 35, pp. 303-316 (1995).
23. N. Mihala, J. Bódi, Á. Gömöry and H. Süli-Vargha, *J. Pept. Sci.*, **7**, 565 (2001); https://doi.org/10.1002/psc.352
24. A. Vegotsky, K. Harada and S.W. Fox, *J. Am. Chem. Soc.*, **80**, 3361 (1958); https://doi.org/10.1021/ja01546a042
25. T. Munegumi, Y.-Q. Meng and K. Harada, *Chem. Lett.*, **17**, 1643 (1988); https://doi.org/10.1246/cl.1988.1643
26. T. Munegumi and K. Harada, *Peptide Chem.*, **1990**, 75 (1991).
27. T. Munegumi, T. Yamada, Y.-Q. Meng and K. Harada, *Peptide Chem.*, **1991**, 179 (1992).
28. T. Munegumi, K. Kawashima and K. Aramaki, *Peptide Chem.*, **1992**, 137 (1993).
29. T. Munegumi, Y.-Q. Meng and K. Harada, *Asian J. Chem.*, **26**, 4716 (2014); https://doi.org/10.14233/ajchem.2014.16185
30. T. Munegumi, K. Akao, Y. Kawatu, T. Yamada and K. Harada, *Asian J. Chem.*, **26**, 6541 (2014); https://doi.org/10.14233/ajchem.2014.16493
31. T. Munegumi and T. Yamada, *Int. J. Polym. Sci.*, **2017**, 8364710 (2017); https://doi.org/10.1155/2017/8364710
32. G.W. Anderson and A.C. McGregor, *J. Am. Chem. Soc.*, **79**, 6180 (1957); https://doi.org/10.1021/ja01580a020
33. G.W. Anderson, J.E. Zimmerman and F.M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964); https://doi.org/10.1021/ja01063a037