Development of a Facile Method for Polypeptide Synthesis. Synthesis of Bòvine Pancreatic Trypsin Inhibitor (BPTI)

Saburo AIMOTO,* Noriko MIZOGUCHI, Hironobu HOJO, and Shoko YOSHIMURA
Institute for Protein Research, Osaka University, Yamakaoda, Suita, Osaka 565
(Received July 27, 1988)

A new method for the preparation of pure polypeptides based on the minimun-protection strategy is described. Its effectiveness was demonstrated by a synthesis of bovine pancreatic trypsin inhibitor (BPTI). Protected peptide segments of Z-[Arg(Tos)^{1}, Asp(O*c*Hex)^{3}, Cys(Acm)^{5}, Glu(O*c*Hex)^{7}]-BPTI(1—13), Boc-[Cys(Acm)^{14,30}, Lys(Z)^{15,26}]-BPTI(14—36), and [Cys(Acm)^{38,51,55}, Lys(Z)^{41,46}]-BPTI(37—58) were prepared by modifying the products obtained by solid-phase peptide syntheses. The protected peptides corresponding to BPTI(14—36) and BPTI(37—58) were coupled by an active ester method to give protected BPTI(14—58). Next, protected BPTI(1—13) was coupled to the BPTI(14—58) by an active ester method after removing the Boc group from the protected BPTI(14—58). All of the protecting groups on BPTI(1—58) were removed, and the resulting peptide was oxidized to give the native form of BPTI in a very pure form. The synthetic BPTI was shown to be identical with the native BPTI in an elution experiment on ion-exchange chromatography and in its inhibitory activity. The new method proved to be very effective for a rapid preparation of pure polypeptide.

Solid-phase peptide synthesis permits effective peptide-chain elongation on a solid support within a short time.[1] However, purification of the final product becomes more difficult with increasing peptide size. On the other hand, a liquid-phase synthesis of a polypeptide is extremely tedious, even for an experienced peptide chemist. To overcome these problems, several approaches have been proposed.[2-5]

We have reported a facile method[6] for the preparation of a cyclic peptide using a protected peptide derived from the product obtained by a solid-phase method. The results suggested that the method could be applied to the preparation of polypeptides.

In this paper, we describe a novel method for the preparation of polypeptide. Its effectiveness was demonstrated by the synthesis of a well-studied small protein, BPTI. This method should be applicable to the syntheses of other peptides of this size.

Results and Discussion

Preparation of Protected Peptide Segments. As shown in Fig. 1, BPTI was divided into three peptide segments: BPTI (1—13), BPTI (14—36), and BPTI (37—58). To avoid racemization during segment coupling, their carboxyl-terminal amino-acid residues were designed to be a glycine or proline residue.

Since BPTI (1—13) contains aspartyl and glutamyl residues, selective protection of α- and ω-carboxyl groups of this peptide segment was realized by the solid-phase method involving N^{α}-(3-nitro-2-pyridinesulfenyl) amino acids (Npys-amino acid) and N^{α}-(9-fluorenylmethyloxycarbonyl) amino acids (Fmoc-amino acid) as shown in Fig. 2. Preparation of the Npys-prolyl resin **6** and chain elongation by using Npys-amino acid were carried out by Matsueda's method.[7] Fmoc-Cys(Acm) was introduced according

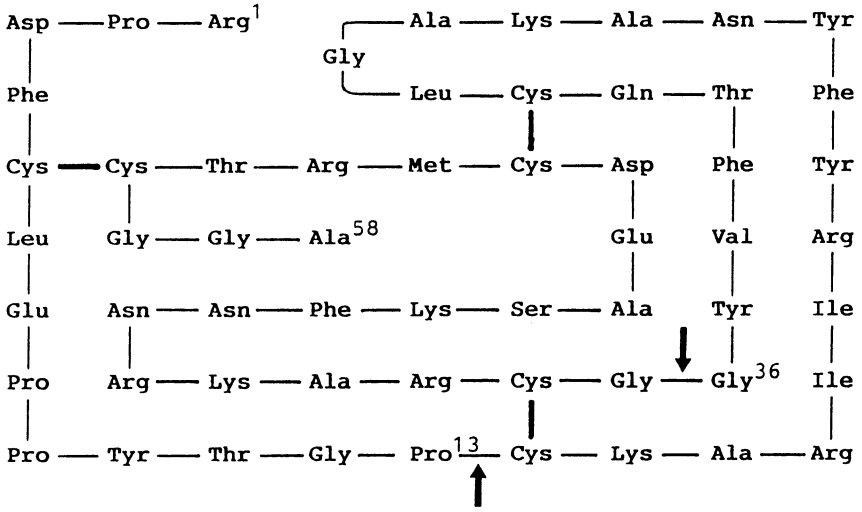

Fig. 1. Primary sequence of bovine pancreatic trypsin inhibitor (BPTI). Arrows indicate the sites where segment coupling was carried out.

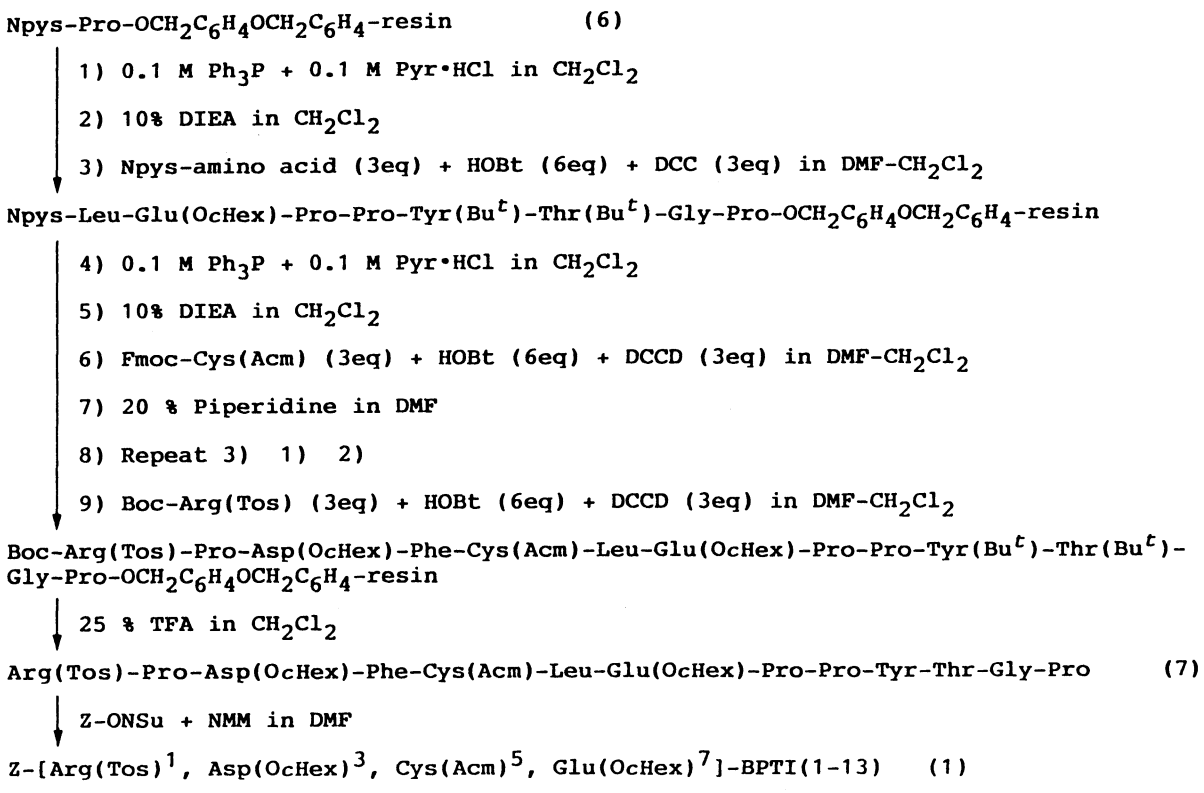

Fig. 2. Synthetic scheme of partially protected BPTI(1—13) **1**

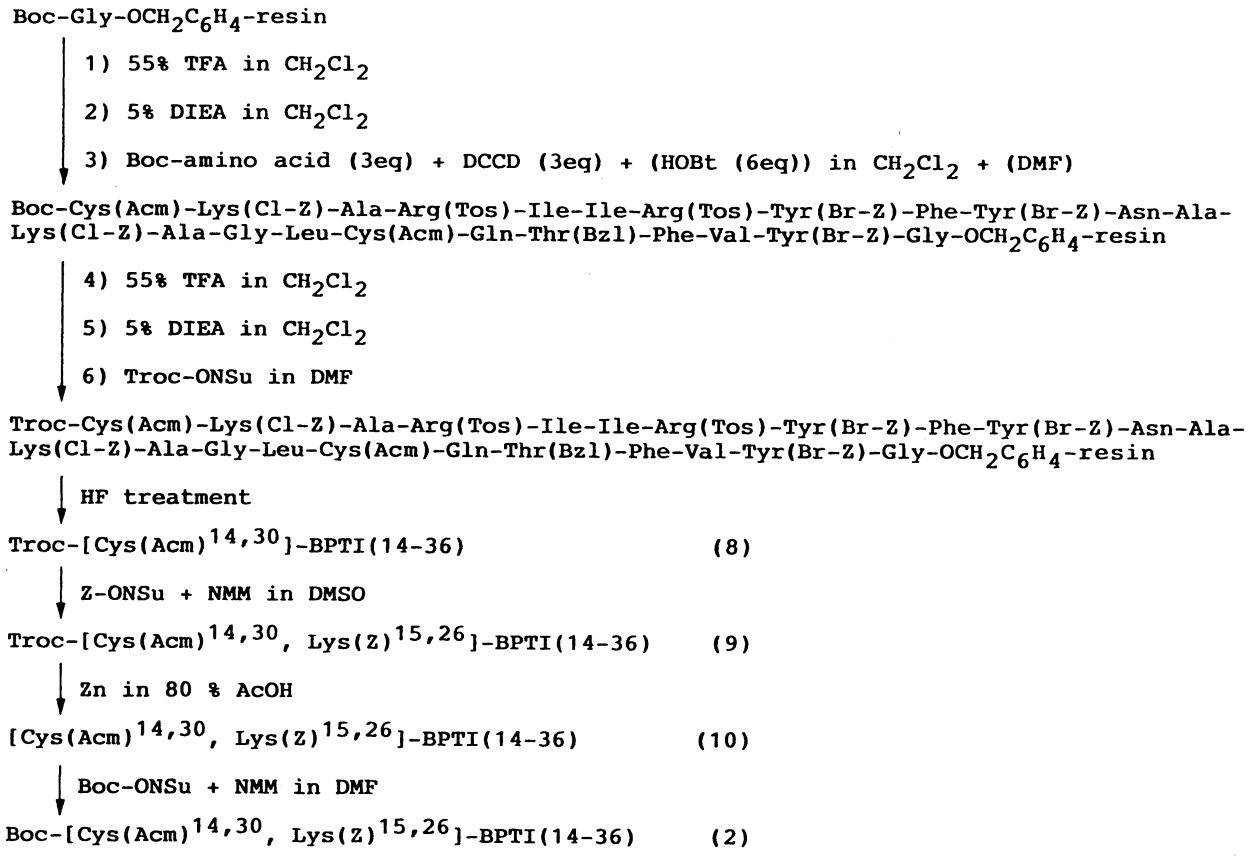

Fig. 3. Synthetic scheme of partially protected BPTI(14—36) **2**.

to Chan et al. with slight modifications.[8] After completion of the peptide chain assembly, the protected peptide resin was treated with 25% trifluoroacetic acid (TFA) in CH_2Cl_2 to obtain protected peptide **7**. This peptide was hydrophobic, but very soluble in aqueous acetonitrile and easily purified by reversed-phase high-pressure liquid chromatography (HPLC). To the amino terminal of the purified peptide, the benzyloxycarbonyl (Z) group was introduced using *N*-(benzyloxycarbonyloxy)succinimide (Z-ONSu) in the presence of 4-methylmorpholine (NMM) as a base to give partially protected BPTI(1—13) **1** in a yield of about 30% based on crude peptide **7**. Confirmations of the protected peptides were carried out by a combination of amino acid analysis and fast atom bombardment (FAB) mass spectrometry.

Since BPTI(14—36) contains no side-chain carboxyl group, the protected BPTI (14—36) **2** was prepared by a solid-phase method involving N^{α}-(*t*-butoxycarbonyl) amino acids (Boc-amino acid). To the Boc-glycyl resin, which was prepared by Gisin's method,[9] Boc-amino acids were successively introduced by the standard Merrifield method,[1] in which the completion of each coupling reaction was checked by a Kaiser test.[10] After completion of the chain elongation, the terminal amino group was blocked by an acid-stable 2,2,2-trichloroethoxycarbonyl (Troc) group[6,11-13] to realize the selective protection of α- and ω-amino groups (Fig. 3). Treatment of the protected peptide resin with anhydrous hydrogen fluoride gave Troc-peptide **8**, which was converted to peptide **9** by a treatment with Z-ONSu in the presence of NMM. Treatment of peptide **9** with zinc dust in aqueous acetic acid gave peptide **10**, which was purified on HPLC. To the generated α-amino group of peptide **10**, a Boc group was introduced by a treatment with *N*-(*t*-butoxycarbonyloxy)succinimide (Boc-ONSu) in the presence of NMM to obtain partially protected BPTI (14—36) **2**. The yield of peptide **2** was as low as 3%, based on crude peptide **8**. Peptides **9**, **10**, and **2** tended to form gels in aqueous acetonitrile. This seemed to be responsible for the low recovery yield of the peptide after purification on HPLC.

Partially protected BPTI (37—58) **3** was prepared using the same strategy adopted for the synthesis of protected peptide **2** (Fig. 4). The yield of peptide **3** was about 30%, based on crude peptide **11**.

Segment Coupling of Partially Protected Peptides. Protected BPTI (1—58) **5** was prepared according to the scheme shown in Fig. 5. Peptide **2** was converted to the corresponding active ester in the presence of sevenfold molar excess of *N*-hydroxysuccinimide (HONSu) with a fivefold molar excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSCI·HCl). The ester-formation reaction was monitored by HPLC and FAB mass measurements. The ester was precipitated with the aid of ether, then washed successively with ethyl acetate, dioxane, and water and dried. The obtained powder was dissolved in dimethyl sulfoxide (DMSO) and peptide **3** and NMM were added. The resulting solution was stirred overnight at room temperature. The product, isolated by HPLC, was

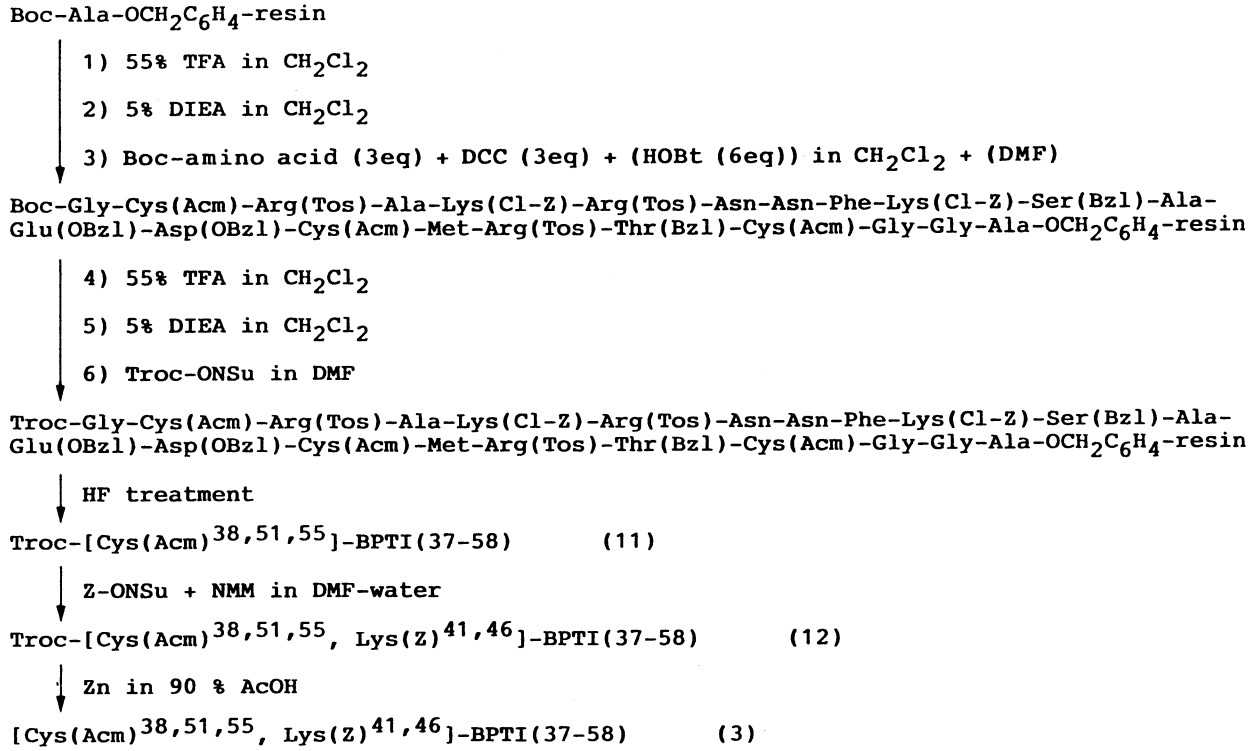

Fig. 4. Synthetic scheme of partially protected BPTI(37—58) **3**.

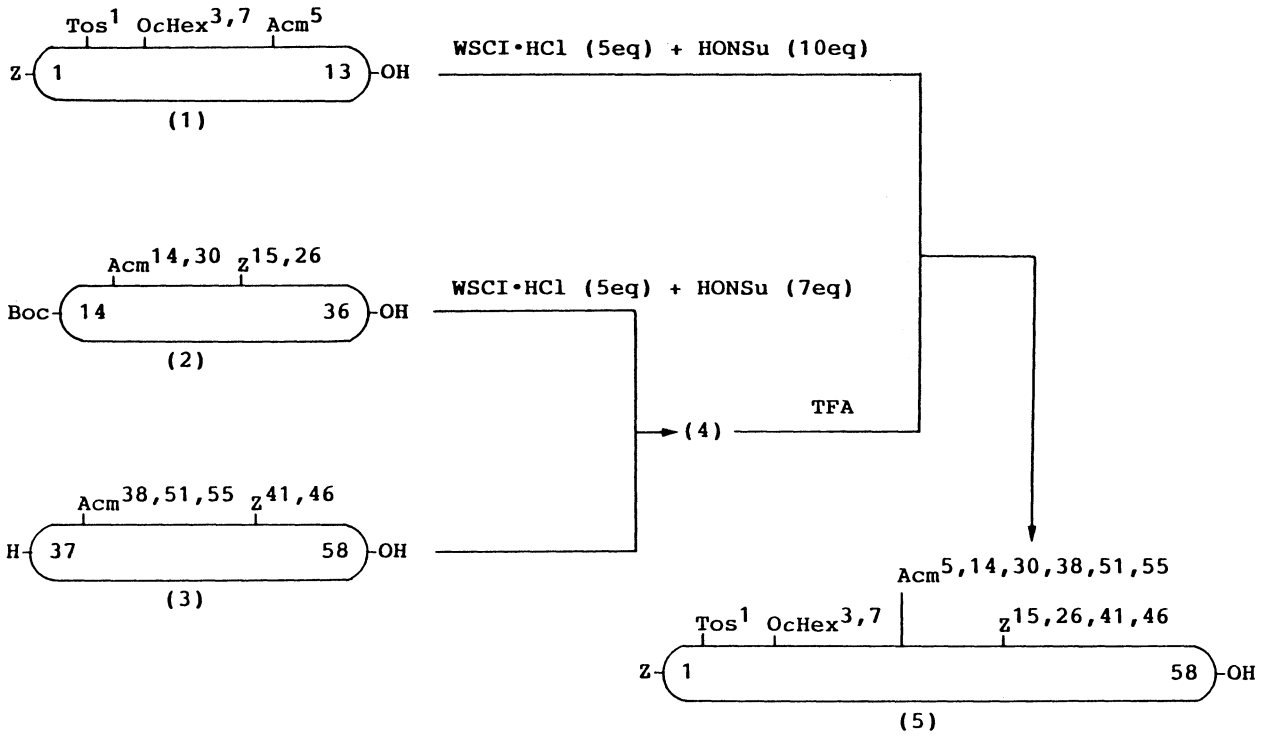

Fig. 5. Synthetic route leading to protected BPTI(1—58) **5** by segment coupling.

confirmed by amino acid analysis to be the desired product **4**, in a yield of about 50%. The active ester of peptide **1**, which was prepared as described above, and the amino-component peptide, which was obtained by TFA treatment of peptide **4**, were mixed in a 2 to 1 molar ratio in DMSO containing 5% NMM, and stirred overnight. Judging from the HPLC profile of the reaction mixture, the amino component reacted almost completely with the active ester to give a product. The amino acid analysis data of the product agreed with the theoretical values expected of protected BPTI (1—58) **5**. Though the segment coupling proceeded almost completely, the yield of the purified peptide **5** was about 30%. The low recovery yield seems to be due to the adsorption of the product on a reversed-phase column during the isolation process.

Generation of the Native Form of BPTI from Peptide 5 and Its Characterization. Protected BPTI (1—58) **5** was treated successively with anhydrous hydrogen fluoride, $Hg(AcO)_2$, and dithiothreitol (DTT) according to the procedure shown in Fig. 6. The reduced form of synthetic BPTI was oxidized in the presence of 1,2-dithiane-4,5-diol (the oxidized form of DTT) according to the method described by Creighton.[14] The native form of synthetic BPTI, thus generated, was adsorbed on TSK gel CM-5PW and eluted with a $AcONH_4$ (pH 8.6) linear gradient (0.01—1 M† $AcONH_4$), as shown in Fig. 7. The main product was eluted at the same position as that of native BPTI. The amino acid analysis data of the product agreed

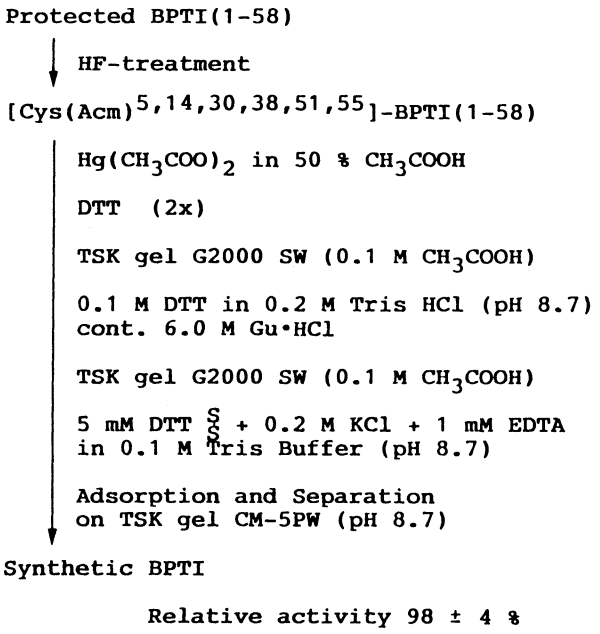

Fig. 6. Operation procedure leading to the native form of synthetic BPTI from protected BPTI(1—58) **5**. DTT^{S}_{S} indicates the oxidized form of DTT.

well with that of native BPTI (Table 1). Furthermore, the product had practically the same inhibitory activity (98±4%) against trypsin as native BPTI. These data show that highly pure BPTI was successfully synthesized.

Evaluation of the Newly Developed Method. Each of the protected peptide segments **1**, **2**, and **3** could be

†1 M=1 mol dm^{-3}.

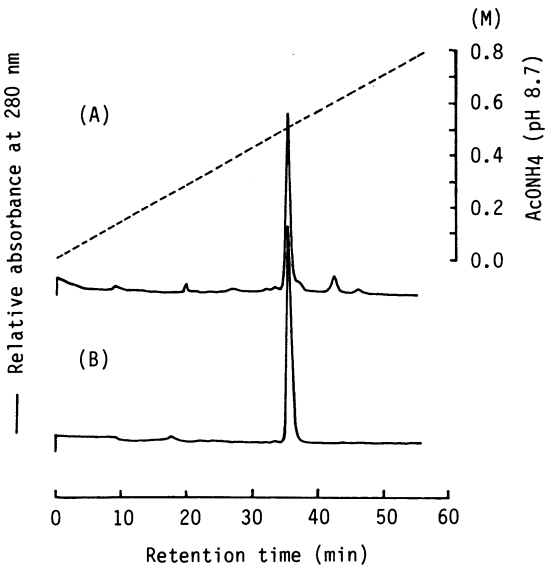

Fig. 7. Ion-exchange chromatogram of synthetic BPTI on TSK gel CM-5PW. (A) The elution profile of crude synthetic BPTI obtained by oxidation with DTT^{S}_{S}. (B) The elution profile of native BPTI. Broken line indicates the concentration of $AcONH_4$ in the buffer.

Table 1. Amino Acid Composition of Synthetic BPTI

	Synthetic BPTI	Native BPTI	Expected
Asp	5.33	5.18	5
Thr	2.98	2.94	3
Ser	1.19	1.03	1
Glu	3.29	3.22	3
Pro	4.02	4.08	4
Gly	6.16	6.14	6
Ala	6	6	6
1/2·Cys	5.11	5.26	6
Val	1.10	1.07	1
Met	0.94	0.96	1
Ile	1.24	1.28	2
Leu	2.07	2.07	2
Tyr	3.96	4.06	4
Phe	4.00	4.06	4
Lys	3.82	3.99	4
Arg	5.84	5.88	6

easily prepared within 2 to 3 weeks starting from the corresponding amino-acyl resins, and the total synthesis of BPTI was accomplished within 2 months. Therefore, the newly developed method enabled a very rapid polypeptide synthesis, which has a very important practical advantage. The minimum-protection strategy worked well on segment coupling. When NMM was used as a base, undesirable side reactions, such as acylation of alcoholic or phenolic hydroxyl groups, were not observed during segment coupling. Furthermore, the elution profile of the native form of BPTI (Fig. 7) revealed that a main product was eluted at the same retention time as native BPTI, and that the peak width of the main product was the same as that of the native one. These facts suggest that all the procedures involved in the synthesis of BPTI proceeded almost perfectly to give highly pure protected BPTI (1—58). Consequently, the pure final product, the native form of synthetic BPTI, could be obtained without employing affinity chromatography. One problem which did arise was the low recovery yield of peptide after HPLC purification. Other than this, we found our method to be useful for the preparation of pure polypeptides of this size.

Materials and Methods

All the amino acids used were of the L-configuration, except for glycine. Boc-amino acids and Bz-Arg-*p*NA·HCl were purchased from Peptide Institute Inc. (Minoh, Japan). Npys-amino acids except for Npys-Glu(O*c*Hex) and Npys-Asp(O*c*Hex), Npys-Cl, Fmoc-Cys(Acm), and *p*-alkoxybenzyl alcohol resin were purchased from Kokusan chemical Works, Ltd. (Tokyo, Japan). Trypsin (bovine pancreas) and bovine pancreatic trypsin inhibitor were purchased from Sigma Chemical Co. Troc-Cl and *trans*-1,2-dithiane-4,5-diol were purchased from Aldrich Chem. Co. *p*-Guanidinobenzoic acid *p*-nitrophenyl ester was purchased from Nacalai Tesque, Ltd. (Kyoto, Japan). All solvents used were of guaranteed grade. High-performance liquid chromatography (HPLC) was carried out on a handmade column of the YMC-Gel ODS S5 100 Å A-type (8×300 mm) (Yamamura Chemical Laboratory Co., Ltd., Kyoto, Japan). Amino acid analysis was carried out using a Jasco amino acid analysis system after acid hydrolysis of a peptide with 4 M methanesulfonic acid (Pierce Chemical Co.) at 110 °C for 24 h. Mass measurement was carried out using a Jeol double-focusing mass spectrometer JMS-HX100, equipped with a fast atom bombardment ion source. Sonication was carried out using a Branson Model B-220. The melting points were determined by the capillary method and are given as uncorrected values. Optical rotations were determined with a Perkin-Elmer Model 241 polarimeter. Abbreviations used are those recommended by IUPAC-IUB: *J. Biol. Chem.*, **247**, 977 (1972). Additional abbreviations: Acm, acetamidomethyl; Cl-Z, 2-chlorobenzyloxycarbonyl; Bzl, benzyl; Br-Z, 2-bromobenzyloxycarbonyl; O*c*Hex, cyclohexyl ester; DCHA, dicyclohexylamine.

Synthesis

Troc-ONSu. Troc-Cl (2.12 g, 10 mmol) and HONSu (1.27 g, 11 mmol) were mixed in dioxane (20 ml) in the presence of triethylamine (TEA) (1.82 ml, 13 mmol) at room temperature for 2 h. The solution was dried, and the product was dissolved in ethyl acetate, washed successively with diluted hydrochloric acid and water, and crystallized from a mixture of ethyl acetate and hexane to obtain Troc-ONSu (2.21 g, 76.0%), mp 105.5—106.0 °C. Found: C, 29.00; H, 2.00; N, 4.79; Cl, 36.48%. Calcd for $C_7H_6O_5NCl_3$: C, 28.94; H, 2.08; N, 4.82; Cl, 36.62%.

Npys-Glu(O*c*Hex)·DCHA. This compound was prepared from Npys-Cl and Glu(O*c*Hex) in the presence of TEA according to a method of Matsueda and Walter.[15] Npys-Glu(O*c*Hex)·DCHA; mp 150—151 °C, $[\alpha]_D^{23}$ +42.7° (*c* 1, ethanol). Found: C, 59.32; H, 7.92; N, 9.94%. Calcd for $C_{28}H_{44}O_6N_4S$: C, 59.55; H, 7.85; N, 9.92%.

Npys-Asp(OcHex)·DCHA. This compound was prepared from Npys-Cl and Asp(OcHex) in the presence of TEA according to a method of Matsueda and Walter.[14] Npys-Asp(OcHex)·DCHA; mp 157—158 °C, $[\alpha]_D^{23}$ +18.9° (*c* 1, ethanol). Found: C, 58.62; H, 7.77; N, 10.13%. Calcd for $C_{27}H_{42}O_6N_4S$: C, 58.87; H, 7.69; N, 10.18%.

Npys-Pro-OCH$_2$C$_6$H$_4$-O-CH$_2$C$_6$H$_4$-Resin (6). Npys-Pro (3.61 g, 13.4 mmol) was coupled to *p*-alkoxybenzyl alcohol resin (5 g, 0.67 meq OH/g) in the presence of 4-dimethylaminopyridine (DMAP) (1.64 g, 13.4 mmol) and dicyclohexylcarbodiimide (DCC) (2.76 g, 13.4 mmol) in *N*,*N*-dimethylformamide (DMF) (30 ml) at room temperature for 14 h. The resin, thus obtained, was treated twice with acetic anhydride (4 ml) in pyridine (20 ml) at room temperature for 30 min and gave 6.3 g of resin **6**. The proline content in the resin was determined to be 0.58 mmol g^{-1} by measuring the absorbance of the solution at 338 nm (ε=6330) after treatment of the resin with a dichloromethane solution containing 0.1 M triphenylphosphine in the presence of 0.1 M pyridine hydrochloride.[7]

Arg(Tos)-Pro-Asp(OcHex)-Phe-Cys(Acm)-Leu-Glu (OcHex)-Pro-Pro-Tyr-Thr-Gly-Pro (7). Npys-Pro resin (**6**) (0.86 g, 0.5 mmol) was placed in a reaction vessel, and the synthesis was carried out manually according to the following protocol by using Npys derivatives of Gly, Thr(Bu^t), Tyr(Bu^t), Pro, Glu(OcHex), Leu, Phe, and Asp(OcHex): (1) washing, CH_2Cl_2 (3×1 min); (2) deprotection, 0.1 M triphenylphosphine and 0.1 M pyridine hydrochloride/CH_2Cl_2 (1×1 min, 2×30 min); (3) washing, CH_2Cl_2 (3×1 min); (4) washing, 2-propanol (2×1 min); (5) washing, CH_2Cl_2 (3×1 min); (6) neutralization, 10% DIEA/CH_2Cl_2 (v/v) (3×1.5 min); (7) washing, CH_2Cl_2 (3×1.5 min) (8) coupling, Npys-amino acid (1.5 mmol) and 1-hydroxybenzotriazole (HOBt) (3 mmol)/DMF(5 ml) and DCC (1.5 mmol)/CH_2Cl_2 (5 ml) (1×1 h); (9) washing, DMF (3×1 min); (10) washing, CH_2Cl_2 (3×1 min); (11) washing, 2-propanol (3×1 min). Cys(Acm) was introduced to the peptide by using an Fmoc derivative under the coupling conditions used for Npys-amino acid; then, a peptide resin was washed successively with DMF, 2-propanol, and CH_2Cl_2. After that, the resin was treated as follows: (1) washing, CH_2Cl_2 (2×1 min); (2) washing, DMF (2×1 min); (3) deprotection, 20% piperidine/DMF (v/v) (1×1 min, 1×20 min); (4) washing, DMF (3×1 min); (5) washing, CH_2Cl_2 (3×1.5 min). The resin was coupled with Npys-Phe. Arg(Tos) was introduced by using its Boc derivative, and washed with DMF, CH_2Cl_2, and 2-propanol. The weight of the peptide resin, thus obtained, was 1.59 g. The whole resin was treated with 25 ml of 25% TFA/CH_2Cl_2 (v/v) for 30 min at room temperature. The resin was removed by filtration, and the filtrate was concentrated to dryness under reduced pressure, leaving a powder (720 mg). This crude product was purified on HPLC with the acetonitrile concentration increasing from 30% to 80% in 0.05% aq TFA at a flow rate of 2 ml min^{-1} and, gave 217.7 mg of compound **7**. Amino acid analysis: $Asp_{0.97}Thr_{1.01}Glu_{0.89}$ $Gly_{1.00}$ 1/2$Cys_{0.14}$ $Leu_{0.92}$ $Tyr_{0.93}Phe_{0.97}Arg_{0.27}Pro_{3.98}$. Found: *m*/*z* 1881.0 $(M+H)^+$ Calcd for $C_{90}H_{130}N_{17}O_{23}S_2$: 1881.0.

Z-[Arg(Tos)^1, Asp(OcHex)^3, Cys(Acm)^5, Glu(OcHex)^7]-BPTI(1—13) (1). Peptide **7** (23.4 mg, 12.4 μmol) and Z-ONSu (8 mg, 32.2 μmol) were dissolved in DMF (1 ml) containing NMM (10 μl), and the solution was stirred for 2 h. A product was precipitated by the addition of ether to the solution, and washed successively with ethyl acetate and dioxane. The product was further purified on HPLC under the same conditions as compound **7** and gave 14.0 mg of compound **1**. Amino acid analysis: $Asp_{0.98}Thr_{0.95}Glu_{0.97}Pro_{3.77}$ $Gly_{1.00}$ 1/2$Cys_{0.29}Leu_{0.99}Tyr_{1.00}Phe_{0.98}Arg_{0.36}$. Found: *m*/*z* 2014.9 $(M+H)^+$. Calcd for $C_{98}H_{136}N_{17}O_{25}S_2$: 2015.1.

Troc-Cys(Acm)-Lys-Ala-Arg-Ile-Ile-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys(Acm)-Gln-Thr-Phe-Val-Tyr-Gly (Troc-[Cys(Acm)^{14,30}]-BPTI(14—36)) (8). Boc-Gly-OCH$_2$C$_6$H$_4$-resin (Gly 0.66 mmol g^{-1}, 2 g) was placed in a reaction vessel, and the synthesis was carried out manually using the Boc derivatives of Tyr(Br-Z), Val, Phe, Thr(Bzl), Gln, Cys(Acm), Leu, Gly, Ala, Lys(Cl-Z), Asn, Arg(Tos), and Ile. The schedule was as follows: (1) washing, CH_2Cl_2 (2×1 min); (2) deprotection, 55% TFA/CH_2Cl_2 (1×5 min, 1×15 min); (3) washing, CH_2Cl_2 (2×1 min); (4) washing, 33% dioxane/CH_2Cl_2 (1×2 min); (5) washing, CH_2Cl_2 (1×1 min); (6) washing, 33% dioxane (2×1 min); (7) washing, CH_2Cl_2 (3×1 min); (8) neutralization, 5% DIEA/CH_2Cl_2 (2×5 min); (9) washing, CH_2Cl_2 (3×1 min); (10) coupling, 3 equiv of Boc-amino acid/CH_2Cl_2 and 3 equiv of DCC/CH_2Cl_2 (1×120 min) except for Boc derivatives of Gln, Asn and Arg(Tos). For the coupling of Boc-Gln, Boc-Asn, or Boc-Arg(Tos), 3 equiv of Boc-amino acid and 6 equiv of HOBt/DMF, and 3 equiv of DCC/CH_2Cl_2 (1×120 min); (11) washing, CH_2Cl_2 (1×1 min); (12) washing, 2-propanol (1×2 min); (13) washing, CH_2Cl_2 (1×1 min); (14) washing, 2-propanol (1×2 min); (15) washing, CH_2Cl_2 (1×1 min); (16) Kaiser test.[10] When an unreacted amino group was detected at step 16, steps 9 to 16 were repeated. The terminal Troc group was introduced by adding 3 equiv of Troc-ONSu/DMF (2×150 min) after step 9. The weight of the protected-peptide resin, thus obtained, was 5.46 g. An aliquot of the resin (754 mg) was treated with anhydrous HF containing 10% anisole (v/v) at 0 °C for 70 min to give 374 mg of crude product **8** after lyophilization. This crude peptide was used for the preparation of compound **9** without further purification. An aliquot of the product was purified on HPLC, giving peptide **8**, which was confirmed as follows. Found: *m*/*z* 3002.0 $(M+H)^+$. Calcd for $C_{133}H_{201}N_{35}O_{34}S_2Cl_3$: 3001.6. Amino acid analysis: $Asp_{0.93}Thr_{0.90}Glu_{0.97}Gly_{1.89}Ala_{3.00}$1/2$Cys_{0.00}Val_{0.85}Ile_{0.89}$ $Leu_{0.91}Tyr_{2.74}Phe_{1.84}Lys_{1.86}$ $Arg_{1.78}$.

Troc-[Cys(Acm)^{14,30}, Lys(Z)^{15,26}]-BPTI(14—36) (9). Crude compound **8** (255 mg, ca. 85 μmol) was dissolved in DMSO (4 ml) containing NMM (0.4 ml). Z-ONSu (106 mg, 425 μmol) was added to the solution which was allowed to react for 3 h with stirring. Ether was added to the solution to precipitate a peptide, and the precipitate was washed with ethyl acetate and then dioxane. The product was purified on HPLC with the acetonitrile concentration increasing from 20% to 80% in 0.05% aq TFA at a flow rate of 2 ml min^{-1} and gave 43 mg of compound **9**. Found: *m*/*z* 3270.2 $(M+H)^+$. Calcd for $C_{149}H_{213}N_{35}O_{38}S_2Cl_3$: 3269.7. Amino acid analysis: $Asp_{0.98}$ $Thr_{0.96}Glu_{1.01}Gly_{1.92}Ala_{3.00}$1/2$Cys_{0.00}Val_{0.89}Ile_{0.87}Leu_{0.97}$ $Tyr_{2.73}Phe_{1.93}Lys_{1.89}Arg_{1.82}$.

[Cys(Acm)^{14,30}, Lys(Z)^{15,26}]-BPTI(14—36) (10). Compound **9** (40 mg) was dissolved in 80% aq acetic acid (6 ml) and zinc dust (100 mg) was added under mild sonication over 4 h at room temperature. The remaining zinc dust was removed by centrifugation. Water was added to the supernatant and the precipitate which formed was washed with water and dried, leaving a powder (35 mg). This powder was purified by HPLC under the conditions used for compound **9** to obtain compound **10** (24 mg); Found: *m*/*z* 3096.1 $(M+H)^+$. Calcd

for $C_{146}H_{212}N_{35}O_{36}S_2$: 3095.7. Amino acid analysis: $Asp_{0.98}Thr_{0.97}Glu_{0.98}Gly_{1.98}Ala_{3.00}1/2Cys_{0.81}Val_{0.84}Ile_{0.77}Leu_{0.97}Tyr_{2.76}Phe_{1.90}Lys_{1.86}Arg_{1.80}$.

Boc-[Cys(Acm)^{14,30}, Lys(Z)^{15,26}]-BPTI(14—36) (2). Compound **10** (21.7 mg) was dissolved in DMSO (0.5 ml) containing NMM (10 μl), and Boc-ONSu (20 mg) was added to the solution which was then stirred for 3 h at room temperature. Ethyl acetate was added to the solution to form a precipitate, which when washed with dioxane and freeze-dried gave a powder (19.0 mg). This was purified by HPLC under the conditions used for compound **7** and 8.2 mg of compound **2** was obtained. Found: *m/z* 3196.0 $(M+H)^+$. Calcd for $C_{151}H_{220}N_{35}O_{38}S_2$: 3195.8. Amino acid analysis: $Asp_{0.98}Thr_{0.95}Glu_{0.96}Gly_{1.97}Ala_{3.00}1/2Cys_{0.60}Val_{0.86}Ile_{0.80}Leu_{0.96}Tyr_{2.73}Phe_{1.90}Lys_{1.87}Arg_{1.83}$.

Troc-Gly-Cys(Acm)-Arg-Ala-Lys-Arg-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys(Acm)-Met-Arg-Thr-Cys(Acm)-Gly-Gly-Ala (Troc-[Cys(Acm)^{38,51,55}]-BPTI(37—58)) (11). Boc-Ala-$OCH_2C_6H_4$-resin (Ala 0.53 mmol g^{-1}, 4.2 g) was placed in a reaction vessel, and peptide-chain elongation and introduction of a Troc group were carried out according to the schedule used for compound **8**, using Boc derivatives of Gly, Cys(Acm), Thr(Bzl), Arg(Tos), Met, Asp(OBzl), Glu(OBzl), Ser(Bzl), Lys(Cl-Z), Phe, and Asn. The weight of the protected peptide resin was 10.4 g. This resin (4.0 g) was treated with anhydrous HF (40 ml) containing anisole (4 ml) at 0 °C for 70 min and gave 1.16 g of crude peptide **11**, which was purified by HPLC with the acetonitrile concentration increasing from 10 to 45% in 0.05% aq TFA at a flow rate of 2 ml min^{-1}. Freeze-drying of the main fraction gave 414 mg of compound **11**. Found: *m/z* 2761.6 $(M+H)^+$. Calcd for $C_{104}H_{172}N_{38}O_{36}S_4Cl_3$: 2762.3. Amino acid analysis: $Asp_{2.95}Thr_{0.70}Ser_{0.92}Glu_{0.96}Gly_{2.82}Ala_{3.00}1/2Cys_{0.47}Met_{0.93}Phe_{0.94}Lys_{1.95}Arg_{2.95}$.

Troc-[Cys(Acm)^{38,51,55}, Lys(Z)^{41,46}]-BPTI(37—58) (12). Compound **11** (400 mg, 145 μmol) was dissolved in a mixture of DMF (5 ml) and water (2.5 ml). Z-ONSu (361 mg, 1.45 mmol) and NMM (500 μl) were then added, and the solution was stirred for 2 h at room temperature. Methanol (10 ml) was added to the solution to form a precipitate, which was washed with methanol (4×) and then with water (1×); it was then dried to give compound **12** (318 mg). Found: *m/z* 3031.0 $(M+H)^+$. Calcd for $C_{120}H_{184}N_{38}O_{40}S_4Cl_3$: 3030.4. Amino acid analysis: $Asp_{3.02}Thr_{0.74}Ser_{0.95}Glu_{1.00}Gly_{2.82}Ala_{3.00}1/2Cys_{0.24}Met_{0.94}Phe_{0.98}Lys_{1.92}Arg_{2.92}$.

[Cys(Acm)^{38,51,55}, Lys(Z)^{41,46}]-BPTI(37—58) (3). Compound **12** (318 mg, 105 μmol) was dissolved in 90% aq acetic acid (12 ml). Zinc dust (300 mg) was added with mild sonication under a nitrogen atmosphere over 3 h at room temperature. A product was isolated by HPLC with the acetonitrile concentration increasing from 25 to 50% in 0.05% aq TFA at a flow rate of 2 ml min^{-1}; 212 mg of compound **3** was obtained. Found: *m/z* 2856.6 $(M+H)^+$. Calcd for $C_{117}H_{138}N_{38}O_{38}S_4$: 2856.5. Amino acid analysis: $Asp_{3.04}Thr_{0.74}Ser_{0.95}Glu_{0.96}Gly_{3.07}Ala_{3.00}1/2Cys_{0.15}Met_{0.96}Phe_{0.97}Lys_{1.95}Arg_{2.99}$.

Z-[Cys(Acm)^{14,30,38,51,55}, Lys(Z)^{15,26,41,46}]-BPTI(14—58) (4). Compound **2** (7.4 mg, ca 2.2 μmol) was dissolved in DMSO (200 μl). HONSu (1.7 mg, 15 μmol) and WSCI·HCl (1.9 mg, 10 μmol) were added and the solution was stirred for 2 h at room temperature. Ether was added to the solution to form a precipitate, which was washed successively with ethyl acetate (1×), dioxane (1×), and water (2×) and dried. The obtained powder and compound **3** (12 mg, ca. 4.2 μmol) were dissolved in DMSO (100 μl) containing NMM (10 μl), and stirred overnight at room temperature. A product was isolated by HPLC with the acetonitrile concentration rising from 20 to 80% in 0.05% aq TFA to give 6.0 mg of compound **4**. Amino acid analysis of compound **4**: $Asp_{3.92}Thr_{1.61}Ser_{0.92}Glu_{1.98}Gly_{4.99}Ala_{6.00}1/2Cys_{0.57}Val_{0.83}Met_{0.83}Ile_{0.73}Leu_{0.98}Tyr_{2.42}Phe_{2.69}Lys_{3.70}Arg_{4.53}$.

Z-[Arg(Tos)^1, Asp(O*c*Hex)^3, Glu(O*c*Hex)^7, Cys(Acm)^{5,14,30,38,51,55}, Lys(Z)^{15,26,41,46}]-BPTI(1—58) (5). Compound **1** (4.35 mg, ca. 2.2 μmol), WSCI·HCl (0.42 mg, 2.2 μmol) and HONSu (1.1 mg, 10 μmol) were dissolved in DMSO (50 μl), and allowed to react for 3 h at room temperature. Ether was added to the solution and a precipitate of an active ester of compound **1** was formed. Compound **4** (6.0 mg, ca. 1 μmol) was treated with TFA (200 μl) at room temperature for 30 min. Freeze-drying of the solution in a test tube gave a solid mass. To the test tube, the active ester dissolved in DMSO (100 μl) and NMM (5 μl) were added. The resulting solution was stirred overnight at room temperature. A product was isolated from the reaction mixture by HPLC under the conditions used for compound **4** and 2.35 mg of compound **5** was obtained. Amino acid analysis: $Asp_{5.56}Thr_{3.35}Ser_{0.90}Glu_{3.67}Pro_{4.40}Gly_{6.51}Ala_{6.00}1/2Cys_{0.00}Val_{0.83}Met_{0.88}Ile_{0.77}Leu_{2.69}Tyr_{4.28}Phe_{4.50}Lys_{3.63}Arg_{5.36}$.

Generation of the Native Form of a Synthetic BPTI. Compound **5** (1.70 mg, 0.21 μmol) was treated with anhydrous HF (1 ml) containing anisole (0.1 ml) for 70 min at 0 °C. After evaporation of HF and anisole, the peptide was dissolved in 50% aq acetic acid (200 μl), and treated with $Hg(CH_3COO)_2$ (3 mg, 10 μmol) for 3 h at room temperature. DTT (5 mg, 32 μmol) in water (200 μl) was added and the precipitate that formed was removed by centrifugation after 30 min. To the supernatant, DTT (5 mg) was added. The solution was left for 2 h at room temperature, and then peptide was isolated by gel filtration on TSK-gel G2000 SW (7.5×600 mm), using 0.1 M aq acetic acid as a buffer. Fractions containing peptide were freeze-dried to obtain a powder, which was treated with DTT (2 mg, 13 μmol) in 0.2 M Tris·HCl (pH 8.7, 200 μl) containing 6.0 M guanidine hydrochloride under a nitrogen atmosphere for 5 h at room temperature. The solution was acidified by acetic acid, and the peptide was isolated by passing through TSK-gel G2000 SW (0.1 M acetic acid). Peptide-containing fractions were collected, and the peptide was oxidized in a buffer (13.44 ml) containing 0.2 M KCl, 1 mM EDTA, and 5 mM of the oxidized form of DTT after adjusting the pH of the buffer to 8.7 with solid tris(hydroxylmethyl)aminomethane at room temperature for 2 h. The solution was diluted to 150 ml with water. At each time, 50 ml of the solution was adsorbed on TSK-gel CM-5PW (7.5×75 mm) and eluted with a buffer (pH 8.7), increasing the ammonium acetate concentration from 0.01 M to 1 M over 70 min at a flow rate of 1 ml min^{-1}. This procedure was repeated three times. The total amount of the peptide in the main fractions was determined to be 27.6 nmol (13.1%) by amino acid analysis.

As a control experiment, native BPTI (76.9 nmol) was reduced and oxidized under the same conditions. The regenerated BPTI was isolated under the same procedure to obtain the native form of BPTI (11 nmol, 14%).

Inhibitory Activity of Synthetic BPTI. Active trypsin concentration was determined according to Chase and Shaw.[16] The trypsin inhibitor concentration was deter-

mined by amino acid analysis after acid hydrolysis of BPTI by 4 M methanesulfonic acid at 110 °C for 24 h. The remaining tryptic activity was measured according to Kassell by employing N^{α}-benzoyl-L-arginine *p*-nitroanilide (BAPNA).[17] Trypsin (1.69×10^{-5} M, 100 μl) and an appropriate amount of synthetic or native BPTI were added into 3 ml of 0.1 M tris buffer (pH 7.8) containing 0.02 M $CaCl_2$. The resultant solution was incubated for 3 min at 25 °C and then 100 μl of BAPNA solution (10 mg ml^{-1} of DMF) was added. The Δ OD_{405} per min was measured with a Hitachi 124 spectrophotometer. The activity of synthetic BPTI was measured at four different concentrations of synthetic BPTI. The specific activity of synthetic BPTI toward the native one was calculated to be 98±4%.

The authors wish to express their thanks to Professor Yasutsugu Shimonishi and Dr. Toshifumi Takao of the Institute for Protein Research, Osaka University for allowing use of the facilities for mass spectra measurement.

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