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Studies on Peptides. XLIV.[1-3] Synthesis of the Basic Trypsin Inhibitor from Bovine Pancreas (Kunitz and Northrop) by the Fragment Condensation Procedure on Polymer Support

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Five protected peptide subunits which cover the entire 58 amino acid residues of the bovine pancreatic basic trypsin inhibitor, *i.e.*, Z–Arg(NO_2)–Pro–Asp(OBzl)–Phe–Cys(Bzl)–Leu–Glu(OBzl)–Pro–Pro–Tyr–Thr–Gly–OH (I, positions 1—12), Z(OMe)–Pro–Cys(Bzl)–Lys(Z)–Ala–Arg(Tos)–Ile–Ile–Arg(Tos)–Tyr–Phe–Tyr–Asn–Ala–Lys(Z)–Ala–Gly–OH (II, positions 13—28), Z(OMe)–Leu–Cys(Bzl)–Gln–Thr–Phe–Val–Tyr–Gly–Gly–OH (III, positions 29—37), Z(OMe)–Cys(Bzl)–Arg(Tos)–Ala–Lys(Z)–Arg(Tos)–Asn–Asn–Phe–Lys-(Z)–Ser–Ala–Glu(OBzl)–Asp(OBzl)–Cys(Bzl)–Met–Arg(Tos)–Thr–Cys(Bzl)–Gly–OH (IV, positions 38—56), Z(OMe)–Gly–Ala–OH (V, positions 57—58), were successively assembled on the polymer support by means of dicyclohexylcarbodiimide plus N-hydroxysuccinimide. In each step, the Z(OMe) group was deprotected by trifluoroacetic acid and the unreacted amino component on the resin was masked by acetylation. After deblocking of all protecting groups by HF and subsequent oxidation followed by affinity chromatographic purification, a highly active peptide, specific activity 82%, indistinguishable from the natural bovine pancreatic basic trypsin inhibitor, was isolated.

As reported in a series of papers,[1,5-7] the synthesis of five protected peptide subunits which cover the entire 58 amino acid residues of the bovine pancreatic basic trypsin inhibitor (Kunitz and Northrop, BTI)[8-10] has been accomplished as shown in Fig. 1. After assembling these fragments on the polymer support followed by deprotection and purification, we have succeeded in isolating a highly active peptide indistinguishable from the natural bovine pancreatic BTI in disc electrophoresis. Detail account of the synthesis of this protein with a molecular weight ca 6500 by the solid phase peptide fragment condensation procedure is presented in this paper.

Condensation scheme of peptide fragments on the polymer support is illustrated in Fig. 2. First, Z(OMe)–Gly–Ala–OH[1] was esterified onto the bromomethylated copolymer of styrene

1) Part XLIII: H. Yajima, Y. Kiso, and K. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **22**, 1079 (1974).
2) Preliminary communication of this article has appeared in part in the Proceedings of the 9th Symposium on Peptide Chemistry (Ed. by N. Yanaihara, Shizuoka, Japan), 115 (1972).
3) Peptides and their derivatives, except Gly, are of the L-configuration. Abbreviations used are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Z=benzoyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Bzl=benzyl, Tos=*p*-toluenesulfonyl.
4) Location: *Sakyo-ku, Kyoto.*
5) H. Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **22**, 1061 (1974).
6) H. Yajima, Y. Okada, H. Watanabe, and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **22**, 1067 (1974).
7) H. Yajima, N. Mizokami, M. Kiso, T. Jinnouchi, Y. Kai, and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **22**, 1075 (1974).
8) B. Kassell and M. Laskowski Sr., *Biochem. Biophys. Res. Commun.*, **20**, 463 (1965); *idem*, *J. Biol. Chem.*, **242**, 4925 (1967).
9) V. Dlouha, D. Pospisilovia, B. Meloun, and F. Sorm, *Coll. Czech. Chem. Commun.*, **30**, 1311 (1965), **33**, 1363 (1968).
10) J. Chauvet, G. Nouvel, and R. Acher, *Biochim. Biophys. Acta*, **92**, 200 (1964); R. Acher and J. Chauvet, *Bull. Soc. Chim. France*, **1967**, 3954.

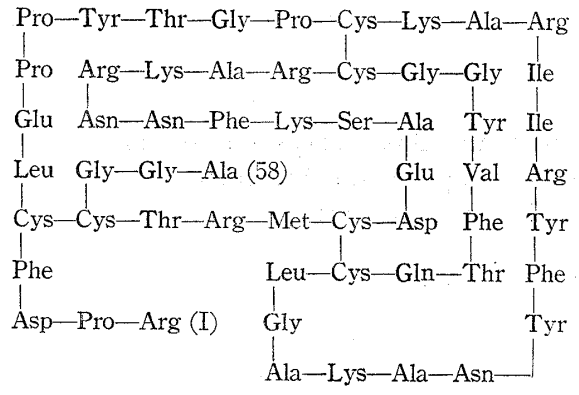

Fig. 1a. Structure of the Basic Trypsin Inhibitor from Bovine Pancreas (Kassell and Laskowski)

and 2% divinylbenzene in the presence of dicyclohexylamine. As we have demonstrated previously,[11,12] this resin seems preferable to the chloromethylated resin, since the esterification of acylpeptides can be achieved at room temperature, while the latter requires long heating in ethanol. The ratios of the peptide and the resin in this esterification step were 1 to 5. By the use of the excess resin, attachment of the peptide to the resin was purposely limitted to avoid the overload of the large peptide on the polymer at the later stage of condensation. Since it seemed impor-

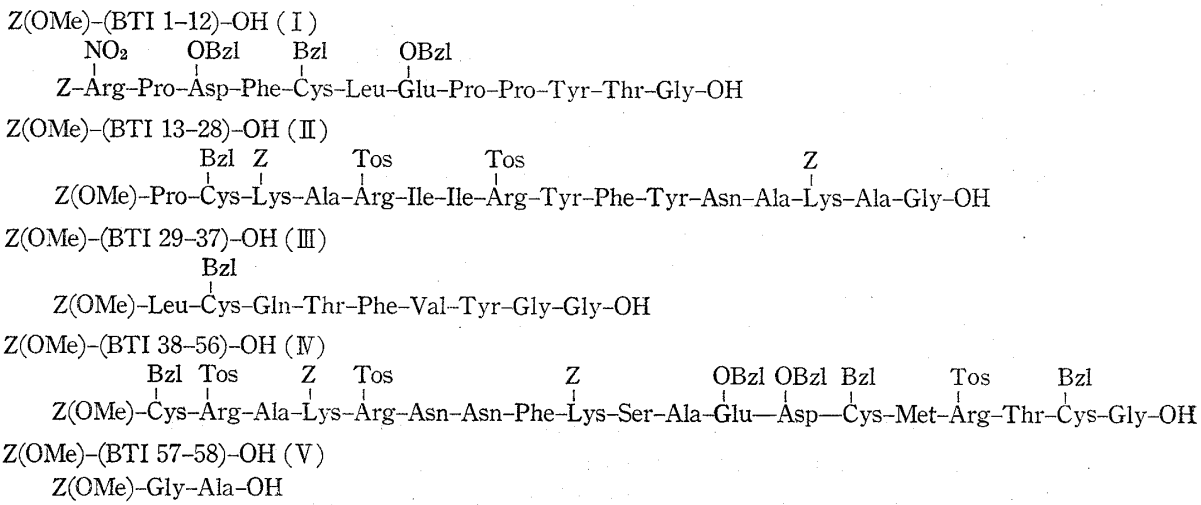

Fig. 1b. Synthetic Subunits of the Basic Trypsin Inhibitor

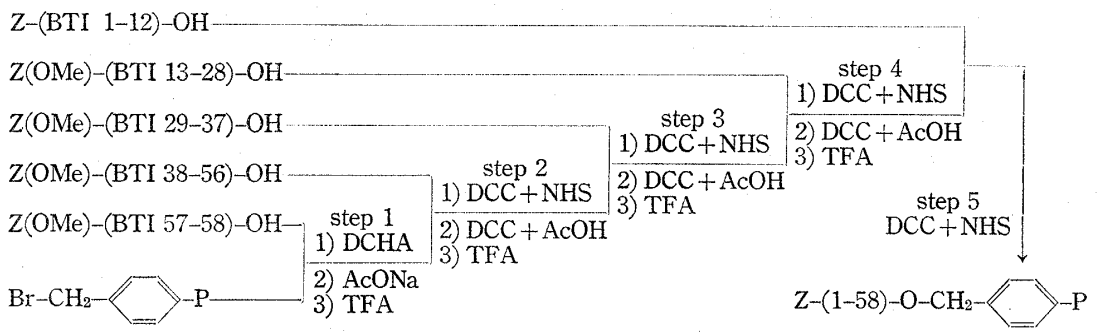

Fig. 2. Condensation Scheme of the BTI Peptide Fragments

tant to carry out every condensation reactions in sterically favorable states as possible. The resin containing the peptide 0.1 mM/g served as the starting material. After esterification, the excess bromomethyl function of the resin was converted to the corresponding acetoxymethyl group by treatment with sodium acetate.[11]

Next, the removal of the α-protecting group from Z(OMe)–Gly–Ala–resin was performed by treatment with 50% trifluoroacetic acid (TFA) in methylenechloride[11,13] for 60 minutes.

11) H. Yajima, H. Kawatani, and H. Watanabe, *Chem. Pharm. Bull.* (Tokyo), **18**, 1333 (1970).
12) H. Yajima and H. Kawatani, *Chem. Pharm. Bull.* (Tokyo), **19**, 1905 (1971).
13) B. Gutte and R.B. Merrifield, *J. Am. Chem Soc.*, **91**, 501 (1969); S. Karlsson, G. Lindeberg, J. Porath, and U. Ragnarsson, *Acta Chem. Scand.*, **24**, 1010 (1970).

This deprotected dipeptide resin, after neutralization with triethylamine, was submitted to the next coupling reaction with the protected nonadecapeptide, Z(OMe)–(BTI 38—56)–OH.[1] Throughout this synthetic work dicyclohexylcarbodiimide (DCC) plus N-hydroxysuccinimide (NHS)[14] was used as the coupling reagent. The amount of the carboxyl component used was increased from 1.5 equivalent to higher ratios as the chainlength increased. Slight modification in ratios of the carboxyl component and the peptide resin was made depending on the coupling efficiency. Each coupling reaction was performed at room temperature for 4 days and a part of the resin was submitted for acid hydrolysis.

In step 2, the condensation reaction between the protected nonadecapeptide and the dipeptide resin proceeded in 75% yield, when 1.5 equivalents of the former component was employed. Since BTI contains the only one residue of Ser, the recovery of Ser in acid hydrolysate was taken as the basis of calculation of the coupling yield.

In the C-terminal portion of BTI, Gly residues are located at positions 37, 56 and 57. Therefore condensation of the large carboxy terminal Gly component and the small dipeptide component had to be performed to avoid the risk of racemization. Despite of such an unfavorable condensation, the coupling reaction proceeded in 75% yield as mentioned above, indicating that the adduct, NHS, was effective in suppressing the acylurea formation, a side reaction of the DCC coupling reaction.

In our preliminary experiments, it was found that esterification of a large peptide, for example the protected nonadecapeptide, onto the bromomethylated resin was unsuccessful. Esterification with a small peptide fragment on the polymer support was used therefore for the first fragment condensation reaction. Occurrence of Gly residue near the C-terminal portion of BTI offered an advantageous feature to this synthetic approach.

In the above coupling reaction, the unreacted H–Gly–Ala–resin was acetylated with acetic acid and DCC to avoid the further incorporation of this sequence to the next coupling reaction. The protected resin, abbreviated as Z(OMe)–(BTI 38—58)–resin, was then treated with TFA and subsequently with triethylamine as described above. This was then submitted to the coupling reaction with the protected nonapeptide, Z(OMe)–(BTI 29—37)–OH.[7] This peptide is a relatively small fragment but less soluble in DMF. The apparent coupling yield stayed around 66%, when 3 equivalents of the carboxyl component and reagents were employed. Therefore 2 equivalents of each were added and the reaction was continued for an additional 2 days. The apparent coupling yield thereby achieved was 82% and addition of more DCC did not improve the yield.

The resulting Z(OMe)–(BTI 29—58)–resin, after acetylation of the unreacted amino component and subsequent deprotection of the Z(OMe) group by TFA, was submitted to the condensation reaction with the protected hexadecapeptide, Z(OMe)–(BTI 13—28)–OH[6] in step 4. As mentioned previously, this peptide is very insoluble in DMF compared to the other fragments. When 4 equivalents of the peptide were employed, some material precipitated during the reaction. From the ratios of Ser and newly incorporated Val or Pro in an acid hydrolysate of the peptide resin thus treated, it can be estimated that the coupling reaction between fragment II and the peptide resin proceeded to about 78% yield.

The Z(OMe)–(BTI 13—58)–resin thus obtained, after acetylation of the unreacted amino function, was treated with TFA followed by triethylamine as described above. The peptide resin hereby obtained was submitted to the final coupling reaction with the N-terminal protected dodecapeptide, Z–(BTI 1—12)–OH[5] in step 5. This peptide is easily soluble in DMF and the apparent coupling yield was nearly 100% or more, when 5 equivalents of the peptide were employed. The result suggested that this dodecapeptide condensed, in part, with the

14) *a*) F. Weygand, D. Hoffmann, and E. Wunsch, *Z. Naturforsch.*, **21b**, 426 (1966); *b*) E. Wunsch and F. Dress, *Chem. Ber.*, **99**, 110 (1966); *c*) G.S. Omenn and C.B. Anfinsen, *J. Am. Chem. Soc.*, **90**, 6571 (1968); *d*) F. Weygand and U. Ragnarsson, *Z. Naturforsch.*, **21b**, 1141 (1968).

Table I. Amino Acid Analysis of Peptide-resin

	Step 1		Step 2		Step 3		Step 4		Step 5	
Gly	1	1.00	2	2.33	4	4.09	5	4.80	6	4.98
Ala	1	1.00	3	3.19	3	3.32	6	5.89	6	4.37
Thr			1	0.86	2	1.67	2	1.68	3	2.64
Arg			3	2.34	3	2.22	5	3.40	6	3.73
Met			1	0.47	1	0.47	1	0.69	1	0.43
Asp			3	2.80	3	3.05	4	4.02	5	4.30
Glu			1	0.96	2	1.87	2	1.82	3	2.89
Ser			1	1.00	1	1.00	1	1.00	1	1.00
Lys			2	1.87	2	1.70	4	3.30	4	3.33
Phe			1	0.84	2	1.84	3	2.58	4	3.33
Tyr					1	0.23	3	1.44	4	2.02
Val					1	0.85	1	0.79	1	0.65
Leu					1	0.81	1	0.70	2	1.89
Ile							2	0.99	2	0.66
Pro							1	0.78	4	4.07
Total	2		21		30		46		58	
Yield		71%		75%		82%		78%		≒100

partially liberated ε-amino function of the Lys residues during the TFA treatment or with the unacetylated amino component. Thus the possibility can not be excluded that during the condensation processes from step 2 to 5, some missing sequence or some branched peptide chain formed on the resin. The apparent coupling yields in these fragment condensation reactions are listed in Table I. The efficiency of the coupling seems to depend not only on the molecular size but also on the solubility of peptides employed as a carboxyl component. In addition, the sterical environment of the amino terminal residue may also influence the efficiency.[14c] NHS is effective in suppressing the acylurea formation during the DCC condensation reaction, but is known to cause a side reaction in the formation of β-Ala-derivative.[15] For this reason, we are examining the use of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline[12,16] or N-isobutoxycarbonyl-2-isobutoxy-1,2-dihydroquinoline[17] as an alternate reagent.

At present experiments, starting from 2 g of the Z(OMe)–Gly–Ala–resin, 2.74 g of the peptide resin was obtained. Though some of the resin was taken off for acid hydrolysis, overall coupling yield on the weight basis was 42%.

The peptide resin was then treated with hydrogen fluoride according to Sakakibara, *et al.*[18] to cleave the peptide from the resin[19] and to remove all protecting groups. Anisole and Met were added as scavengers. The deblocked product would be a mixture of different molecular size resulted from the fragment condensation reactions. Gel filtration was first applied for the purification. As shown in Fig. 3, two main peaks were observed when the eluent was examined by the ultraviolet (UV) absorbancy at 280 mμ. The front peak which presumably contained the desired compound was not a symmetrical peak and therefore the eluates were divided into 6 fractions. Activity could be generated if the right compound

15) M. Low and L. Kisfaludy, *Acta Chim. Acad. Sci. Hung.*, **44**, 61 (1965); H. Gross and L. Bilk, *Tetrahedron*, **24**, 6935 (1968); F. Weygand, W. Steglich, and N. Chytil, *Z. Naturforsch.*, **23b**, 1391 (1968).
16) B. Bellow and G. Malek, *J. Am. Chem. Soc.*, **90**, 1651 (1968).
17) Y. Kiso and H. Yajima, *Chem. Commun.*, **1972**, 942; Y. Kiso, Y. Kai, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **21**, 2507 (1973).
18) S. Sakakibara and Y. Shimonishi, *Bull. Chem. Soc. Japan*, **38**, 1412 (1965).
19) J. Lenard and A.B. Robinson, *J. Am. Chem. Soc.*, **89**, 181 (1967).

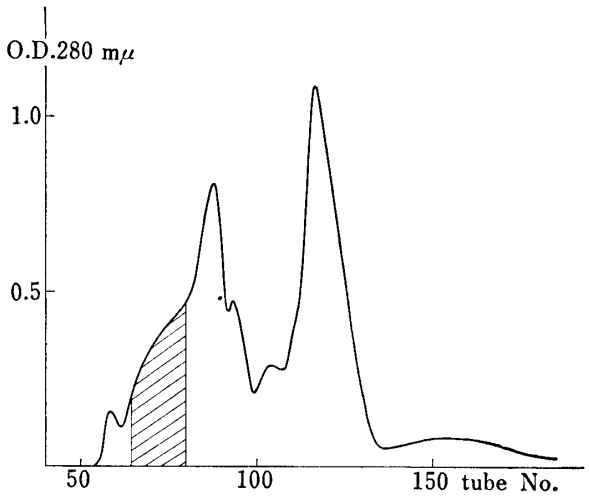

Fig. 3. Chromatographic Pattern of the Deblocked Peptide on Sephadex G-25

mn: 2.2×135 cm, solvent: 0.1N AcOH, 3 g eachecuol/tub

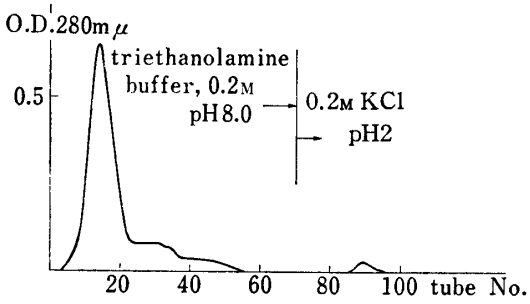

Fig. 4. Chromatographic Pattern of the Synthetic BTI on Trypsin-sepharose Column

was oxidized by air to form the three disulfide bridges.[20,21] According to Sorm, *et al.*[20] each fraction was diluted with a solution of sodium acetate and exposed to air at pH 4.5 for 3 days. References indicated that among various conditions for reoxidation of the reduced natural BTI, such slow oxidation was preferable to the rapid oxidation by air stream.[20,21] The BTI activity was measured, according to Walsh[22] and Sorm, *et al.*[20] using tosylarginine methyl ester as substrate. Fraction II (shadow in Fig. 3) was the only fraction which was active and the rest was inactive. As can be seen from the chromatographic pattern, large

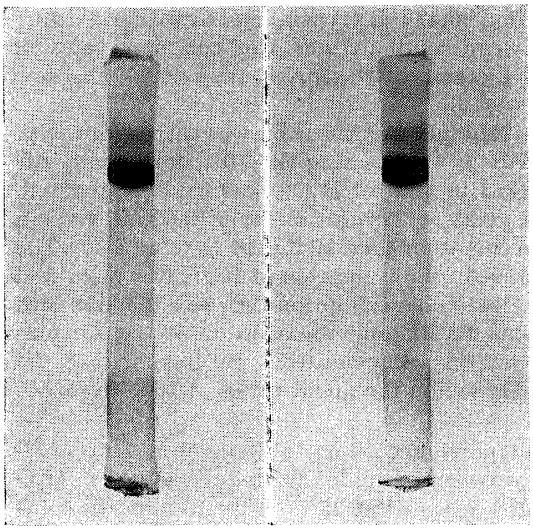

Fig. 5. Disc Electrophoresis of the Synthetic BTI (left) and Natural BTI (right) Polyacrylamide Gel (7.5%, pH 9.4), Tris-glycine Buffer pH 2.3, Migration was from Top (cathod) to Bottom at 4° for 60 min (5 mA/tube) (stained by Amido Black 10B)

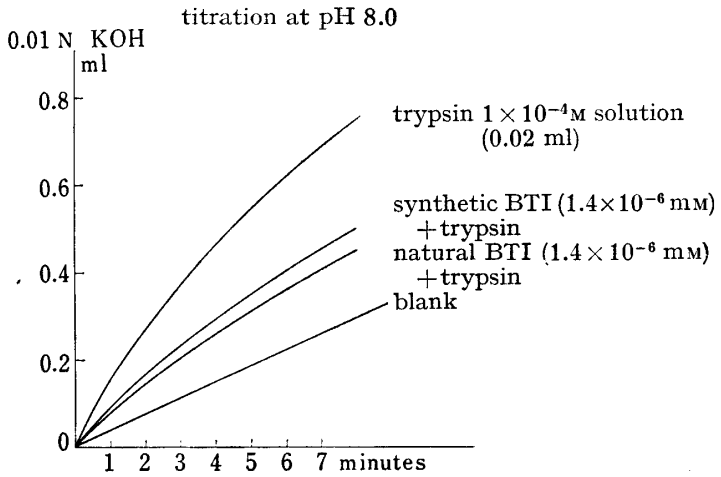

Fig. 6. Assay of the Synthetic BTI

20) D. Pospisilova, B. Meloun, I. Fric, and F. Sorm, *Coll. Czech. Chem. Commun.*, **32**, 4108 (1967).
21) F.A. Anderer and S. Hornle, *J. Biol. Chem.*, **241**, 1568 (1966); R.A. Goldman, I. Snir, G. Blauer, and M. Rigbi, *Arch. Biochem. Biophys.*, **121**, 107 (1967).
22) K.A. Walsh in "Methods in Enzymology," Vol. 19, ed. by G.E. Perlamnn and L. Lorand, Academic Press, New York, 1970, p. 42.

inactive fractions might result not only from wrong sequences accumulated on the polymer but also from the formation of incorrect pair of disulfide bridges.

The active fraction was further purified by affinity chromatography through trypsin-sepharose column according to Chauvet and Acher.[23] The column was first eluted with triethanolamine buffer and then the desired compound was eluted with 0.2 M potassium chloride at pH 2.0 as shown in Fig. 4. After desalting by Sephadex G-25, the peptide obtained migrated in the disc electrophoresis as the natural BTI as shown in Fig. 5. Upon acid hydrolysis, it contained the constituent amino acids in ratios predicted by theory, except for Ile, which gave only one mole after usual 24 hr acid hydrolysis as mentioned previously.[6,9] When the specific activity of this purified peptide was compared with the natural BTI using tosyl-arginine methyl ester as a substrate,[24] a specific activity of 82% was found as shown in Fig. 6. Data presented here may justify the conclusion that our synthetic BTI possesses a high degree of homogeneity and activity.

The yield of the purified deblocked active peptide was 7.5% starting with the blocked peptide-resin. Further effort in improving the yield of BTI is underway in our laboratory.

Experimental

Assay of the BTI Activity by the Spectrophotometric Procedure——Inhibition of Tryptic hydrolysis of tosylarginine methyl ester (TAME) was measured according to the procedure described by Sorm, *et al.*[20] and Walsh.[22]

Reagents: Substrate, 0.01 M TAME (37.889 mg in 10 ml of H_2O). Buffer, 0.05 M Tris(hydroxymethyl)-aminomethane (Tris)—HCl, pH 8.1.

Trypsin Solution: 1×10^{-4} M (Sigma 2 cryst. from bovine pancreas Type 1, No. T-8003, 24 mg of trypsin was dissolved in 10 ml of 0.001 M HCl containing 22 mg of $CaCl_2$).

Standard Solution of BTI: 1.11×10^{-5}M (Sigma from bovine pancreas Cryst, Type I–P, No. T-0256. 0.13 mg of BTI was dissolved in 1.8 ml of 0.05 M Tris buffer).

For the determination of the BTI activity, 0.01 M TAME (0.3 ml) and 0.05 M Tris-HCl buffer (2.7 ml) were mixed and placed in a cuvet (3 ml, 1 cm). Trypsin solution (0.02 ml, trypsin concentration 2×10^{-6} mM) and BTI solution (0.18 ml, BTI concentration 2×10^{6} mM) were combined and this combined solution (0.02 ml) was added to a solution of TAME prepared above. Increase of the absorbancy at 247 mμ was determined at each 30 sec intervals for 5 min.

Determination of the BTI Activity by pH Stat Titration[24]——The pH stat titration was carried out with a Radiometer Titrator (type TTT 1) and a titrigraph (Type SBR 2). Expecting the 70% inhibition, the standard BTI solution, 3.5×10^{-5}M (0.91 mg of BTI was dissolved in 4 ml of H_2O), was freshly prepared. 0.01 M TAME (0.9 ml) and H_2O (8.1 ml) were placed in a bath at 25°. Trypsin solution (0.02 ml, trypsin concentration 2×10^{-6} mM) and BTI solution (0.04 ml, BTI concentration 1.4×10^{-6} mM) were added. The free carboxyl group released was titrated with 0.01 N KOH at pH 8.0. Consumption of KOH was measured and the specific activity was calculated from the difference of alkaline consumption between the standard trypsin solution (0.02 ml, trypsin concentration 2×10^{-6} mM) plus TAME and the solution of trypsin plus BTI plus TAME. Since TAME is saponified at pH 8, the base line was compensated. By this method, the specific activity of the standard BTI employed was $99.2 \fallingdotseq 100\%$, based on the molar ratios of trypsin (M.W. 24000)[25] and BTI (6500).[8–10]

Preparation of Trypsin-Sepharose——Affinity column was prepared according to Chauvet and Acher.[23] Sepharose 2 B (67 ml, Pharmacia) was washed with H_2O, and cyanogen bromide (325 mg) dissolved in H_2O (26 ml) was added. The pH was adjusted to 11 by titration with 1 N NaOH at 25°. After 15 min, the solution was filtered and the activated sepharose was washed with cold H_2O and cold 0.1 M $NaHCO_3$. This was then suspended in 0.1 N $NaHCO_3$ and trypsin (325 mg, Sigma) was added. The mixture was gently stirred at 4° for 20 hr. The sepharose was collected by filtration, washed succesively with 0.1 M $NaHCO_3$, 1 M NaCl, 0.1 M AcOH+1 M NaCl, 1 M NaCl and H_2O. For the estimation of the bound protein, 3.96 mg of dry sample was submitted for acid hydrolysis. Asp 0.146 μM (22 residues), Ser 0.216 μM (34 residues) and Gly

23) J. Chauvet and R. Acher, *FEBS Letters*, 23, 317 (1972).
24) C.F. Jacobsen, J. Leonis, K. Lindestrom-Lang, and M. Ottesen, in "Methods of Biochemical Analysis," Vol. 4, ed. by D. Glick, J. Wiley Interscience, New York, 1957, p. 171; L. Goldstein, in "Methods in Enzymology," Vol. 19, ed. by G.E. Perlmann and L. Lorand, Academic Press, New York, 1970, p. 950.
25) K.A. Walsh and H. Neurath, *Proc. Natl. Acad. Sci. U.S.*, 52, 884 (1964); O. Mikes, V. Holeysovsky, V. Tomasek, and F. Sorm., *Biochem. Biophys. Res. Commun.*, 24, 346 (1966).

0.202 μM (25 residues) were detected. From these values, 72 mg of bound trypsin/g of sepharose (based on the molecular weight of trypsin 24000[25]) was detected; yield 15%. The trypsin activity was 31%, determined by the pH stat titration.

Z(OMe)-Gly-Ala-Resin——The bromomethylated copolymer of styrene and 2% divinylbenzene (Br 1.3 mM/g) was prepared as described previously.[11] A mixture of this resin (15.4 g, Br 20 mM), Z(OMe)–Gly–Ala–OH[1] (1.24 g, 4 mM) and dicyclohexylamine (0.72 ml, 4 mM) in DMF (60 ml) was shaken at room temperature for 48 hr. The resin, after washing with DMF, H_2O, MeOH and methylene chloride (each 50 ml × 3), was again shaken in DMF (60 ml) in the presence of AcOH (2.4 ml) and triethylamine (5.5 ml) at room temperature for 48 hr and then washed successively with 4 solvents (50 ml × 3 each) as stated above. A part of the resin was submitted for acid hydrolysis: Gly content 0.10 mM/g, esterification yield 71%.

Z(OMe)-(BTI 38—58)-Resin——Z(OMe)–Gly–Ala–resin (2.0 g, peptide content 0.2 mM) prepared above was treated with 50% TFA in methylene chloride (20 ml) containing anisole (1 ml) at room temperature for 60 min, collected by filtration and washed with methylene chloride, DMF, 10% triethylamine in DMF, DMF, 10% triethylamine in DMF, DMF and methylene chloride (20 ml × 3 each). In the case of washing with 10% triethylamine in DMF, the resin was shaken for 10 min before filtration.

The peptide resin was then shaken in DMF containing Z(OMe)–(BTI 38—56)–OH[1] (1.04 g, 0.3 mM), DCC (103 mg, 0.5 mM) and NHS (35 mg, 0.3 mM) for 3 days at room temperature. The resin was washed with DMF, methylene chloride, AcOH and methylene chloride (20 ml × 3 each). A part of the resin was submitted for amino acid analysis. The results are listed in Table I. The resin was then shaken in methylene chloride (40 ml) in the presence of AcOH (0.01 ml) and DCC (21 mg) for 2 hr at room temperature and washed with methylene chloride, AcOH, DMF and methylene chloride (20 ml × 3 each).

Z(OMe)-(BTI 29—58)-Resin——Z(OMe)–(BTI 38—58)–resin obtained above was treated with 50% TFA in methylene chloride (16 ml) containing anisole (1 ml) at room temperature for 60 min and the resin was washed as described above. The deprotected resin was shaken in DMF (40 ml) containing Z(OMe)–(BTI 29—37)–OH[7] (750 mg, 0.6 mM), DCC (247 mg, 1.2 mM) and NHS (69 mg, 0.6 mM) at room temperature for 4 days. The ratios of Ser and Val in an acid hydrolysate of this peptide resin was 66%. After addition of Z(OMe)–(BTI 29—37)–OH (500 mg, 0.4 mM), DCC (165 mg, 0.8 mM) and NHS (46 mg, 0.4 mM), shaking was continued for an additional 2 days. The resin was washed as described above and a part of which was submitted to the amino acid analysis. The result was listed in Table I.

Treatment of this resin in methylene chloride (10 ml) with AcOH (0.01 ml) and DCC (21 mg) and subsequent washing were performed as described above.

Z(OMe)-(BTI 13—58)-Resin——Deprotection of the Z(OMe)– group from Z(OMe)–(BTI 29—58)–resin was carried out as described above by treatment with 50% TFA in methylene chloride (16 ml) containing anisole (1 ml). After shaking for 60 min, the resin was collected by filtration and washed with 10% triethylamine in DMF and other solvents as described above. This resin was shaken in DMF (50 ml) containing Z(OMe)–(BTI 13—28)–OH[6] (2.03 g, 0.8 mM), DCC (330 mg, 1.6 mM) and NHS (92 mg, 0.8 mM) at room temperature for 4 days and then washed with DMF, methylene chloride, AcOH, DMF, dimethyl sulfoxide and methylene chloride (20 ml × 3 each). A part of the resin was acid hydrolized and the result was listed in Table I. Treatment of the resin with AcOH (0.01 ml) and DCC (21 mg) was carried out in essentially the same manner as described above and the resin was washed with methylene chloride, AcOH, DMF and methylene chloride (20 ml × 3 each).

Z-(BTI 1—58)-Resin——Treatment of the peptide resin, Z(OMe)–(BTI 13—58)–resin with TFA and subsequent neutralization were performed in essentially the same manner as described above. The deprotected resin was shaken in DMF (30 ml) containing Z–(BTI 1—12)–OH[5] (1.04 g, 0.5 mM), DCC (206 mg, 1 mM), and NHS (61 mg, 0.5 mM) at room temperature for 5 days and the resin was washed with DMF, methylene chloride, AcOH, DMF and methylene chloride (20 ml × 3). The resin 2.74 g was obtained. Total coupling yield on the weight basis was 42.8%. The sum of the coupling yield in each step based on the Z(OMe)–Gly–Ala–resin was 43.7% and thus both coupling yields can be judged as nearly identical.

Isolation of the Active Peptide——The peptide resin, Z–(BTI 1—58)–resin (560 mg) was treated with HF[18,19] (approximately 10 ml) in the presence of Met (50 mg) and anisole (1 ml) at 5 to 15° for 90 min. The HF was evaporated *in vacuo* and the residue, after drying over KOH pellets *in vacuo* overnight, was dissolved in H_2O (60 ml), which was then washed with ether and lyophilized to give fluffy powder (130 mg containing 50 mg of Met).

This deblocked peptide was dissolved in a solution of 8 M urea–0.05 M Tris-HCl buffer (pH 8, 10 ml) and mercaptoethanol (0.5 ml) was added. The flask was incubated at 40° for 24 hr under nitrogen gas. This solution was then applied to a column of Sephadex G-25 (2.2 × 135 cm), which was eluted with 0.1 N AcOH with a flow rate 6 ml/hr. Individual fractions, 3 g each, were collected and the absorbancy at 280 mμ was determined for each tube. The chromatographic pattern is shown in Fig. 3. Eluates were divived into 6 fractions and the contents of each fraction were combined. Each combined solution was diluted with the same amount of 0.1 N AcONa and after adjusting the pH of the solution to 4.5, was kept in a opened dish on standing at room temperature for 3 days. When inhibition of the tryptic hydrolysis of TAME was measured according to Sorm, *et al.*[20] and Walsh,[22] the activity was found in fraction 64 to 80. The rest

was inactive. This active fraction was then applied to a column of Sephadex G-25 (2.2×135 cm), which was eluted with 0.1 N AcOH as described above; yield white powder 18 mg.

This material was dissolved in 0.2 M triethanolamine buffer (pH 8, 10 ml) and the solution was applied to a trypsin–sepharose column (1.3×24 cm), which was eluted with the same buffer (200 ml) and then with 0.2 M KCl–HCl (pH 2) buffer. Individual fractions, 3 g each, were collected and absorbancy at 280 mμ was determined for each tube. The chromatographic pattern is shown in Fig. 4. The content of fraction 86—95 were combined and the solution, after neutralization with 1 N NaOH, was desalted with a column of Sephadex G-25 (2.2×135 cm). Elution was carried out by using 0.1 N AcOH; yield after lyophilization, white powder 6 mg. Its disc electrophoretic pattern is shown in Fig. 5. Amino acid ratios in an acid hydrolysate (24 hr hydrolysis, the number in bracket indicates the theory): $Asp(5)_{5.27}$ $Thr(3)_{3.02}$ $Ser(1)_{1.00}$ $Glu(3)_{3.09}$ $Pro(4)_{4.69}$ $Gly(6)_{5.87}$ $Val(1)_{0.68}$ $Met(1)_{0.63}$ $Ile(2)_{0.96}$ $Leu(2)_{1.89}$ $Tyr(4)_{2.62}$ $Phe(4)_{4.16}$ $Lys(4)_{4.18}$ $Arg(6)_{5.43}$ (average recovery 80.4%). The specific activity, 82% (average of three assay values, 81.25, 82.69, and 82.14%), was determined from the volume of alkaline consumption (at 5 min titration) using the pH stat titration procedure[24] as shown in Fig. 6, where the same concentration (1.4×10^{-6} mM) with the standard BTI solution was employed.

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