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# Propeptin, a New Inhibitor of Prolyl Endopeptidase Produced by *Microbispora*

## II. Determination of Chemical Structure

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The structure of propeptin, a new inhibitor of prolyl endopeptidase isolated from *Microbispora* sp. SNA-115, was determined. FAB/MS, Edman degradation and amino acid analysis revealed propeptin to be a cyclic polypeptide consisting of 19 common L-amino acids. By FAB/MS and protein chemical methods, the primary sequence of propeptin was determined to be $Gly^1$-Tyr-Pro-Trp-Trp-Asp-Tyr-Arg-$Asp^9$-Leu-Phe-Gly-Gly-His-Thr-Phe-Ile-Ser-$Pro^{19}$, which cyclizes between the $\beta$-carboxyl group of $Asp^9$ and the $\alpha$-amino group of $Gly^1$.

Prolyl endopeptidase (PEP: postproline cleaving enzyme, its latest name is prolyl oligopeptidase: EC 3. 4. 21. 26) cleaves peptide bonds at the carboxyl side of proline residues in biologically active peptides such as vasopressin *etc.*[1~3]. Specific inhibitors of PEP are expected to have anti-amnesic effects and are synthesized as anti-amnesic drugs[4,5].

In the course of screening for a new type of PEP inhibitor from Actinomycete, it was found that *Microbispora* sp. SNA-115 isolated from a soil sample produces a new PEP inhibitor designated as propeptin. In our previous paper, the fermentation, isolation, physicochemical and biological properties of propeptin have been described[6]. In this paper, we report the determination of the primary sequence of propeptin, including the results of DL-amino acid analyses.

As described in our previous paper, propeptin (MW 2295, $C_{113}H_{142}N_{26}O_{27}$) is a polypeptide consisting of 19 amino acids (molar ratio): Asp (2), Thr (1), Ser (1), Gly (3), Ile (1), Leu (1), Tyr (2), Phe (2), His (1), Arg (1), Trp (2), Pro (2)[6]. The molecular weight calculated from its amino acid composition is 18 a.m.u. larger than that determined by FAB/MS. This suggests that propeptin has a certain cyclic structure.

### *N*-Terminal Sequence Analysis of Propeptin

*N*-Terminal sequence analysis was performed by automated Edman degradation. However, no amino acid was detected. This indicated that the terminal $\alpha$-amino group of propeptin is masked and probably forms a cyclic structure.

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Fig. 1. FAB/MS spectra of propeptin (A) and propeptin T (B).

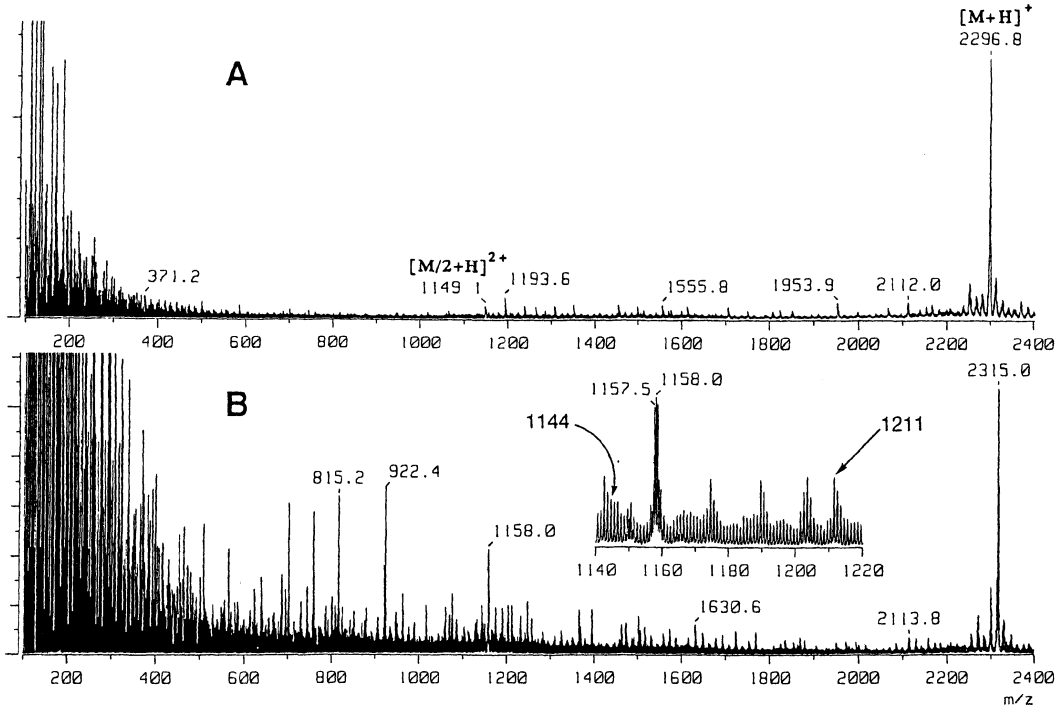
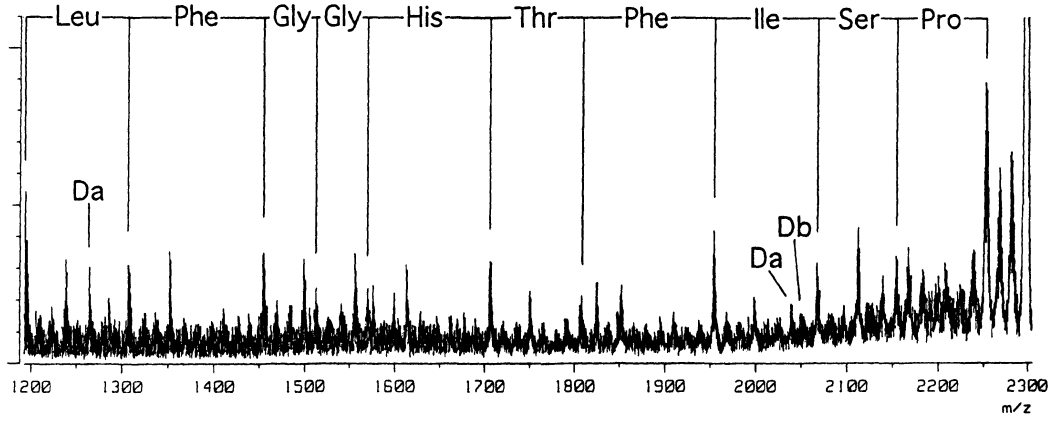



Fig. 2. Amino acid sequence analyses of propeptin by *N*-terminal (A-type) ion series.




## FAB/MS of Propeptin

The FAB/MS spectrum of propeptin is shown in Fig. 1-A. There are many fragment ions between the $[M+H]^+$ ion at *m/z* 2296 and the $[M/2+H]^{2+}$ ion at *m/z* 1149, but almost no useful ions in the mass region lower than that near the doubly charged ion. This characteristic suggests that propeptin consists of a cyclic and chain moiety. Figure 2 shows the mass region between the ion at *m/z* 1193 and the $[M+H]^+$ ion, and the assignments of these fragment ions due to *N*-terminal (A-type) ion series. Based on these assignments, a partial sequence corresponding to the chain moiety in the propeptin molecule was obtained: (HO) Pro-Ser-Ile-Phe-Thr-His-Gly-Gly-Phe-Leu. Of the amino acids

sequenced, Leu and Ile were assigned based on the difference in D-type ions (Da and Db) produced from A-type ions by cleavage of these side chains[7]. This sequence was obtained also by the linked-scan spectrum from $[M+H]^+$ ion at *m/z* 2296 in propeptin (data not shown). The linked-scan spectrum from the fragment ion at *m/z* 1193, which corresponds to the A-type ion of the cyclic moiety of propeptin, provided a partial sequence included in the cyclic moiety: (HO) Arg-Tyr-Asp-Trp-Trp-Pro (data not shown).

### DL-Amino Acid Analysis

Amino acid enantiomers of the propeptin hydrolyzate were differentiated according to their HPLC retention times using a chiral ligand-exchange column[8]. These analytical data for propeptin indicated that the amino acids except glycine had L-configuration.

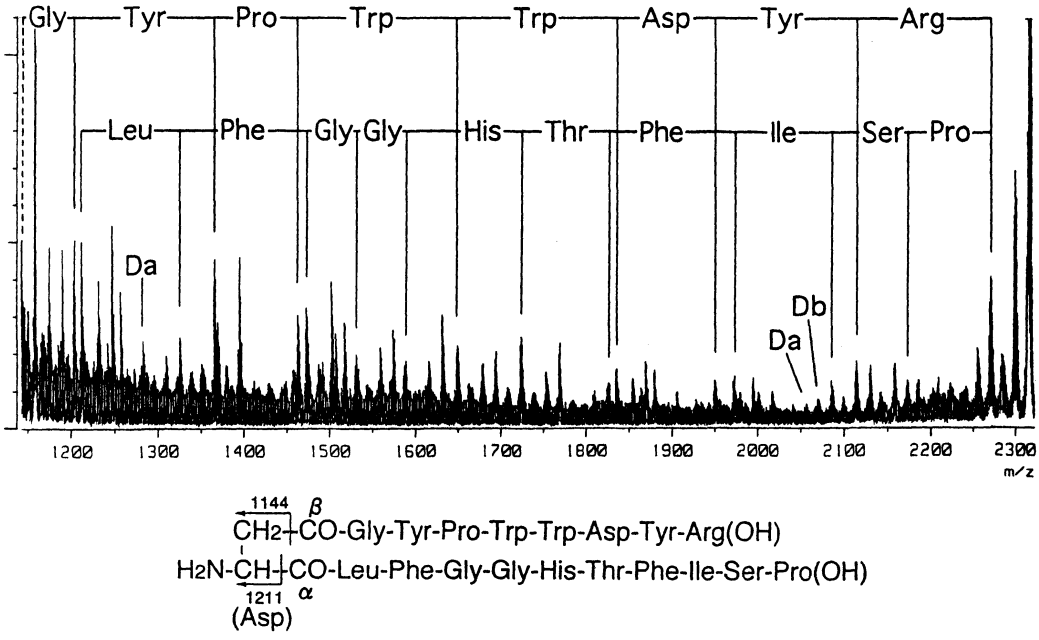
### Propeptin T

In order to elucidate the structure of the cyclic moiety of propeptin, opening of the cyclic structure by trypsin treatment was attempted because an Arg is present in the cyclic moiety of propeptin. A digested product, propeptin T, was obtained as a single peak in HPLC (data not shown). The molecular weight and formula of propeptin T were determined to be 2313 and $C_{113}H_{144}N_{26}O_{28}$ by FAB/MS and HR-FAB/MS, respectively. The amino acid composition of the propeptin T hydrolyzate was completely identical to that of propeptin, (molar ratio): Asp (2.24), Thr (1.02), Ser (1.07), Gly (3.47), Ile (1.00), Leu (1.15), Tyr (2.15), Phe (2.01), His (1.13), Trp (1.39), Arg (1.04) and Pro (2.14). These data show that propeptin T has a chain structure. The UV and IR spectra of propeptin T were almost the same as those of propeptin. The arginyl endopeptidase digestion product of propeptin was also identical with propeptin T in terms of HPLC retention time (data not shown).

### FAB/MS of Propeptin T

The FAB/MS spectrum of propeptin T is shown in Fig. 1-B. Fragment ions are observed over the entire mass region. *N*-Terminal ions among these fragment ions were used to obtain the amino acid sequence. Figure 3 shows the high mass region of propeptin T and the assignments due to the A-type ion series. The chain structure of (HO) Pro to Leu (designated as chain-A) that was sequenced in native propeptin was detected. The characteristic fragment ions differentiating Leu from Ile in chain-A of propeptin T were also observed. In addition to the sequence of chain-A, another sequence of (HO) Arg-Tyr-Asp-Trp-Trp-Pro-Tyr-Gly (designated as chain-B) was obtained. In the latter sequence, *N*-terminus Gly was assigned from a C″-type ion

Fig. 3. Amino acid sequence analyses of propeptin T by *N*-terminal (A-type) ion series and the assignment of two A-type ions (*m/z* 1211 and 1144) produced by the cleavage of the $\alpha$- and $\beta$-carboxyl groups of Asp.



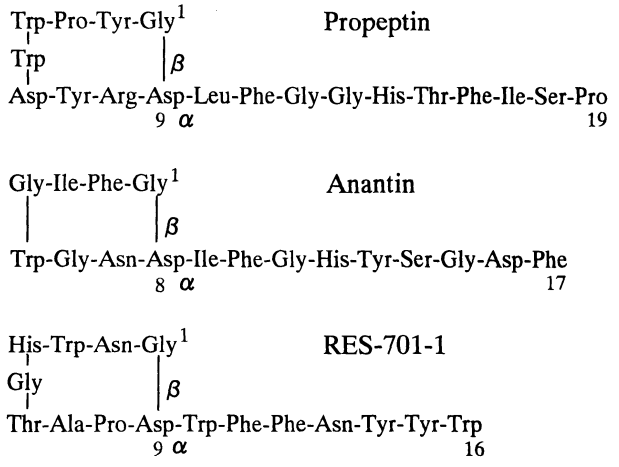
series. Because the fragment ion at *m/z* 1144 which is the A-type ion to assign the *N*-terminus Gly was less abundant. Comparison of the amino acids included in the two chains with the results of amino acid composition analysis suggested that the chains-A and -B bind to an Asp that was not sequenced by FAB/MS; that is, the amino groups of chains-A and -B bind to the $\alpha$- and $\beta$-carboxyl groups of Asp. The connecting positions of chains-A and -B with Asp were deduced based on the analysis of the two fragment ions at *m/z* 1211 and 1144, due to the cleavage of the chain-A or -B residue (see Figs. 1-B and 3). In general, the A-type ion obtained by cleavage at the $\alpha$-carboxyl side of Asp can form a stable immonium-type structure and shows relatively higher intensity than that at the $\beta$-carboxyl side. Apparently, the ion at *m/z* 1211 has higher intensity than that at *m/z* 1144. This shows that in propeptin T, the chain-A is connected to the $\alpha$-carboxyl group, and the chain-B, to the $\beta$-carboxyl group of the Asp. The intense peak representing the fragment ion at *m/z* 1193 observed in the FAB/MS spectrum of propeptin is assigned to the immonium-type ion by cleavage at the $\alpha$-carboxyl side of the Asp (see Fig. 1-A). This finding indicates also that chain-A in propeptin binds to the $\alpha$-carboxyl group of the Asp. *C*-Terminal (Y″-type) ion series analysis of propeptin T showed the presence of two partial amino acid sequences, namely, (HO) Phe-Thr to Leu in chain-A and (HO) Asp to Gly in chain-B. The linked-scan spectrum from $[M+H]^+$ ion (*m/z* 2314) for propeptin T provided the same results as the FAB/MS spectrum (data not shown).

### *N*-Terminal Sequence Analysis of Propeptin T

*N*-Terminal sequence analysis of propeptin T was performed by automated Edman degradation. The sequence of the Leu to Pro (chain-A) was obtained, but the first amino acid did not observed as the known amino acid. This datum supported the elucidated primary sequence of propeptin T by FAB/MS: In the $Asp^9$, the amino group is free and the $\alpha$-carboxyl group is connected to chain-A.

Thus, the unique primary structure of propeptin, which is composed of a cyclic moiety with nine amino acids and a side chain moiety with ten amino acids, was determined (Fig. 4). Recently, cyclic peptides of this type have been found, *e.g.*, anantin[9] and RES-701-1[10], which are an atrial natriuretic peptide receptor antagonist and an endothelin receptor antagonist, respectively. Anantin and RES-701-1 are cyclized between the $\beta$-carboxyl group of $Asp^8$ or $Asp^9$ and the $\alpha$-amino group of $Gly^1$ in the same manner as propeptin. However, there is no sequence similarity among propeptin, anantin and RES-701-1 (Fig. 4). Thus, these peptides do not belong to the same family, but the cyclizing enzyme between the $\beta$-carboxyl group of Asp and the $\alpha$-amino group of Gly may be related. We are now interested in the biosynthesis of these peptides and the cyclizing enzyme in different Actinomycetes species[11].

Fig. 4. Structures of propeptin and related compounds (anantin, RES-701-1).




## Experimental

### Fermentation and Isolation

Strain SNA-115 producing propeptin was cultivated under fermentation conditions and propeptin was isolated as described previously[6].

### Trypsin Treatment of Propeptin

Propeptin (53.8 mg) was dissolved in 4.5 ml of methanol and added to 3.5 ml of 0.1 M Tris-HCl (pH 7.5) and 3 mg of trypsin (dissolved in 1 ml of 0.1 M Tris-HCl (pH 7.5), TPCK treated, 12000 units/mg, SIGMA). It was incubated at 37 °C for 3 hours and purification was carried out by preparative HPLC using Nucleosil $5C_{18}$ (20×250 mm, Senshu pak) with $CH_3CN$-0.1% TFA (35:65). After concentration and lyophilization, propeptin T was obtained as a white powder (27.0 mg).

### Instrumental Analyses

The UV spectrum was measured with a HITACHI U-3210 spectrophotometer. The IR spectrum was recorded on a JASCO DIP-181 digital spectrometer. Amino acid analysis was carried out after hydrolysis with 4 N methane sulfonic acid (Pierce) at 90°C for 24 hours using a

HITACHI L-8500 amino acid analyzer. Chiral reverse HPLC analysis (SUMICHIRAL OA-5000, 4.6 i.d.×150 mm) was conducted to distinguish D- from L-amino acids. Amino acid sequencing was performed on an Applied Biosystems Model 492 automated gas phase sequencer.

Mass Spectrometry

FAB/MS, HR-FAB/MS and FAB/linked-scan measurements were performed using a JEOL JMS-HX110 mass spectrometer. Fast atom bombardment (FAB) was generated using xenon as the primary beam with 6 KeV energy and the ion accelerating voltage was 10 KV. High-energy collision induced dissociation was performed by introducing helium as collision gas until the intensity of the precursor ion was reduced to 1/3 of the initial value. A mixture of glycerol, thioglycerol and a small quantity of hydrochloric acid was used for the FAB ionization matrix.

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