Conformational Studies by ^1H Nuclear Magnetic Resonance of the Trypsin–Chymotrypsin Inhibitor B-III from Peanuts and Its Enzymatically Modified Derivative

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ABSTRACT: The conformation of the peanut inhibitor B-III was investigated by ^1H NMR at 500 MHz. Resonances for about 30% of the residues were assigned and/or identified by conventional and two-dimensional ^1H NMR measurements. The conformation of the modified inhibitor B-III$^{**}_{RS}$, obtained by the cleavage of the peptide bonds of B-III at the first reactive site, Arg(10)–Arg(11), and second reactive site, Arg(37)–Ser(38), was also investigated. Edman degradation was used to identify resonances of the residues adjoining the scissile sites. A comparison of the behavior of the resonances corresponding to Ala(12), Tyr(15), Phe(16), and Thr(37) in B-III and B-III$^{**}_{RS}$ indicated that the only conformational differences between the two proteins were those from the S–S loops containing the two reactive sites. From the similar behavior of Leu(7), Phe(24), His(26), Ala(29), five valine, and four threonine resonances in both proteins, it is suggested that the S–S loops that do not contain a scissile bond (the core region of the inhibitor) were rigid. Thus, no conformational change of the core region was observed upon cleavage of the two reactive sites.

Many protease inhibitors have been isolated from legume seeds, and their amino acid sequences and inhibitory properties have been investigated (Laskowski & Kato, 1980). Five Bowman–Birk type (BBI type)[1] inhibitors (A-I, A-II, B-I, B-II, B-III) have been isolated from peanut seeds (Norioka et al., 1982), and their amino acid sequences also have been determined (Norioka & Ikenaka, 1983a,b). BBI inhibitor from peanuts has unique properties, when compared to those of the other BBI-type inhibitors (Norioka & Ikenaka, 1984). Peanut inhibitor B-III inhibited both trypsin and chymotrypsin activities toward the first site, Arg(10)–Arg(11) bond, and only trypsin activity toward the second site, Arg(38)–Arg(39) bond. The P_1 residue of the first reactive site, Arg(10), does not coincide with the substrate specificity of chymotrypsin. Limited proteolysis of the first reactive site with trypsin or chymotrypsin results in a loss of inhibition for chymotrypsin, but inhibition for trypsin remains as strong as the native inhibitor.

The sequence around the Arg(10)–Arg(11) site of these peanut inhibitors is also unusual, because the P_1' and P_2 positions are occupied by arginine and aspartic acid residues, instead of serine and threonine that are common to nearly all the other BBI-type inhibitors. Moreover, two amino acids, tyrosine and phenylalanine, are inserted in the S–S loop containing this first reactive site.

Because the three-dimensional structure might provide some explanation of the relationship between such a unique inhibitory property and the amino acid sequence of the peanut inhibitor, we have carried out the conformational analysis of peanut inhibitor B-III and its enzymatically modified derivatives by ^1H NMR.

Experimental Procedures

Materials. Peanut inhibitors, A-II and B-III, were prepared as described previously (Norioka et al., 1982). Bovine chymotrypsin (three times crystallized) and TLCK–chymotrypsin were purchased from Sigma Chemical Co. TPCK–trypsin and carboxypeptidase B were products of Worthington Biochemical Corp. Acetic acid-d_4 (100%) was obtained from Commissariat a l'Energie Atomique, and ^2H_2O (100%) was from Merck Sharp & Dohme.

Preparation of B-III$^{**}_{RS}$. B-III$^{**}_{RS}$ was prepared by the limited proteolysis of B-III with TPCK–trypsin (Norioka & Ikenaka, 1983a). Ten milligrams of B-III was incubated with 0.6 mg of TPCK–trypsin in 4.0 mL of diluted acetic acid, pH 3.0, for 48 h at 25 °C. The solution was applied to a Sephadex G-50 column (1.2 × 140 cm) equilibrated with 0.05 M ammonium acetate buffer, pH 7.5, in order to remove TPCK–trypsin as the inhibitor–trypsin complex.

Preparation of B-III$^{*}_{R}$. The limited proteolysis of B-III with TLCK–chymotrypsin was carried out as described previously (Norioka & Ikenaka, 1984). Ten milligrams of B-III was incubated with 0.7 mg of TLCK–chymotrypsin in 2.0 mL of 0.02 M ammonium acetate, pH 4.0, for 1 week at 25 °C. This solution was applied to a SP-Sephadex column (2.5 × 22 cm) equilibrated with 0.02 M sodium acetate–HCl buffer, pH 2.5, and the column was developed with a linear gradient of sodium chloride concentration from 0 to 1.2 M.

Preparation of B-III$^{*}_{S}$. B-III$^{*}_{S}$ was prepared by resynthesis of the Arg(10)–Arg(11) peptide bond in B-III$^{**}_{RS}$ by the complex formation with chymotrypsin as reported previously

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[1] Abbreviations: A-II$^{**}_{RS}$, peanut inhibitor A-II in which two peptide bonds, Arg(10)–Arg(11) and Arg(38)–Ser(39), are cleaved; BBI, soybean Bowman–Birk inhibitor; B-III$^{**}_{RS}$, peanut inhibitor B-III in which two peptide bonds, Arg(10)–Arg(11) and Arg(38)–Ser(39), are cleaved; B-III$^{*}_{R}$, peanut inhibitor B-III in which a peptide bond, Arg(10)–Arg(11), is cleaved; B-III$^{*}_{S}$, peanut inhibitor B-III in which a peptide bond, Arg(38)–Ser(39), is cleaved; CIDNP, chemically induced dynamic nuclear polarization; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; 1D, one-dimensional nuclear magnetic resonance; 2D, two-dimensional nuclear magnetic resonance; TLCK, 1-chloro-3-(tosylamido)-7-amino-2-heptanone; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; TSP, sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$.

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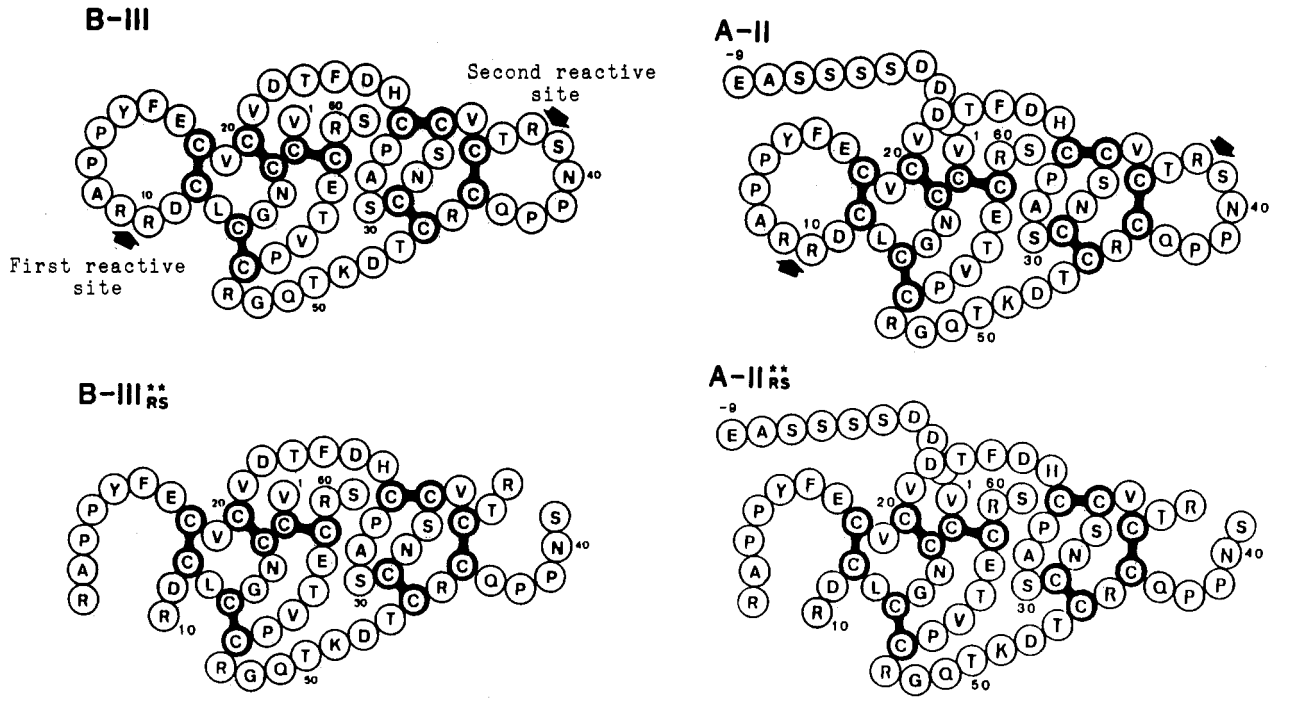

FIGURE 1: Covalent structures of B-III, B-III$^{**}_{RS}$, A-II, and A-II$^{**}_{RS}$ (Norioka & Ikenaka, 1983a,b). Arrows show the reactive sites. The amino acid sequences of A-II and A-II$^{**}_{RS}$ are numbered on the basis of that of B-III.

(Norioka & Ikenaka, 1984). Further purification of B-III$^{*}_{S}$ was carried out by HPLC using a C-18 reversed-phase column (Cosmocil, 5 μm, 0.4 × 30 cm).

Carboxypeptidase B Digestion. B-III$^{**}_{RS}$ was dissolved in 0.05 M Tris-HCl buffer, pH 8.0, and digested with carboxypeptidase B at 25 °C for 24 h at an enzyme to substrate ratio of 1:50 (w/w). The solution was applied to a Sephadex G-50 column (1.2 × 140 cm) equilibrated with 0.05 M ammonium acetate, pH 7.5, for removing the enzyme and amino acids. Released amino acids were detected with a Hitachi amino acid analyzer, Model 835.

Edman Degradation. A manual Edman degradation was carried out by the method of Iwanaga et al. (1969). Two milligrams of the protein was used for each step. After degradation, the sample was applied to Sephadex G-15 column (2 × 50 cm) equilibrated with 0.05 M ammonium acetate, pH 7.5, for removing excess reagents and amino acid derivatives from the protein.

NMR Measurements. ^{1}H NMR spectra were recorded on a JEOL GX-500s spectrometer with a DEC 11-23 computer. Labile protons were substituted with deuterium by heating the sample in ^2H_2O solution at 60 °C for 30 min. Lyophilized samples were redissolved in ^2H_2O containing 5–10% acetic acid-d_4. For pH titrations, dilute ^{2}HCl and NaO^{2}H solutions were used. All pH values are direct pH meter readings of the protein solution with a glass electrode. Chemical shifts were measured relative to an internal reference, TSP. The temperature of the samples was detected by a thermocouple mounted close to the sample tube. Two-dimensional shift correlation spectroscopy (COSY) was obtained at 40 °C with a 90°–τ_1–90° pulse sequence with stepwise-incremented evolution time, τ_1 (Nagayama et al., 1979), and two-dimensional NOE spectroscopy (NOESY) was obtained at 40 °C with 90°–τ_1–90°–τ_m–90° pulse sequence with τ incremented stepwise and τ_m constant at 300 ms (Kumar et al., 1980). The free-induction decay pattern consisted of 2K data points in the t_2 dimension with a sweep width of 5000 Hz and 256 values incremented by the dwell time. The data size was expanded to 512 in the t_1 dimension by zero filling. The spectra have the digital resolutions of 9.8 Hz/point for ω_1 direction and 4.9 Hz/point for the ω_2 direction. The spectra were obtained in the absolute mode. More details concerning the theory and application of two-dimensional NMR can be found in Turner's review (1985) and Bax's book (1982). In all spectra, the peak of ^{1}HO^{2}H was suppressed by homogated decoupling.

Photochemically Induced Dynamic Nuclear Polarization. Photochemically induced dynamic nuclear polarization experiments were performed in the same way as reported by Kaptein (1978). The 0.4 mL of sample solution contained 1.5 mM protein and 0.2 mM 3-*N*-(carboxymethyl)lumiflavin. A CIDNP difference spectrum was obtained by subtracting a dark from a light spectrum; in the light spectrum, the solution was irradiated by an argon ion laser (NEC GLC-3300) with 1.1 W at 488 nm.

Results and Discussion

Identification of Methyl Resonances. Peanut inhibitor B-III contains 13 amino acid residues with methyl groups: one leucine at position 7; two alanines at positions 12 and 29; five threonines at positions 23, 37, 47, 50, and 57; five valines at positions 1, 19, 21, 35, and 56 (Figure 1).

Figure 2 shows the methyl regions of COSY spectra of B-III and B-III$^{**}_{RS}$. On the basis of the coupling pattern and chemical shift of each residue, all methyl resonances in both B-III and B-III$^{**}_{RS}$ were identified as shown in Figure 2. The methyl resonances of leucine were assigned to Leu(7) because B-III contained only one leucine. The methyl signals of alanines and threonines were numbered from low to high field, as shown in Table I. An additional signal appeared close to the A_1 peak in the COSY spectrum of B-III$^{**}_{RS}$, which will be discussed later.

The spectra of B-III and B-III$^{**}_{RS}$ were identical except for the downfield shifts of A_1 and T_2 resonances. As shown in Table I, the shift of A_1 occurred with the cleavage of the first reactive site of B-III and shift of T_2 occurred with the cleavage of the second reactive site of B-III. These shifts were independent. Therefore, the two reactive sites are not next to each other in spatial arrangement of B-III.

Table I: Proton Chemical Shifts of the Methyl Resonances in B-III and Its Modified Derivatives

derivatives	chemical shifts of methyl resonances of Ala and Thr[a] (ppm)					
	A_1	A_2	T_1	T_2	T_3	T_4
B-III	1.460	1.298	1.430	1.390	1.158	1.117
$B\text{-III}^{*}_{R}$	1.357	1.284	1.434	1.386	1.166	1.129
$B\text{-III}^{*}_{S}$	1.454	1.286	1.430	1.264	1.154	1.114
$B\text{-III}^{**}_{RS}$	1.354	1.287	1.424	1.262	1.153	1.112
des-R^{10},R^{38},R^{60},S^{61}-$B\text{-III}^{**}_{RS}$[c]	1.350	1.292	1.418	1.218	1.153	1.111 (pH 3.3)
des-R^{10},R^{38},R^{60},S^{61}-$B\text{-III}^{**}_{RS}$	1.343	1.301	1.425	1.192	1.154	1.113 (pH 4.5)[b]

[a] Shifts were measured relative to TSP, at 40 °C in ^2H_2O solution containing 5% acetic acid-d_4. The resonances of alanines and threonines were numbered from low to high field, respectively. A_1, A_2, and T_2 are assigned to the methyl resonances of Ala(12), Ala(29), and Thr(37), respectively, later. [b] pH was adjusted by NaOD. [c] $B\text{-III}^{**}_{RS}$ in which Arg(10), Arg(38), Arg(60), and Ser(61) are removed.

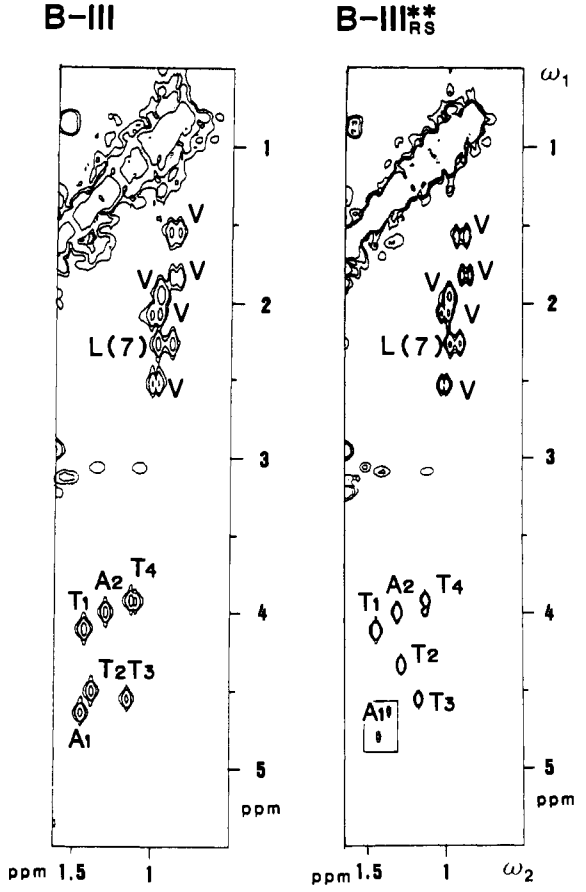

FIGURE 2: Methyl regions of COSY spectra of B-III and $B\text{-III}^{**}_{RS}$. All cross peaks of methyl resonances were identified as shown above for their coupling patterns and chemical shifts, and the methyl resonances of leucine were assigned to Leu(7). The cross peak at ω_1 = 3.9 ppm and ω_2 = 1.1 ppm consists of two signals of threonine. The satellite signals corresponding to alanine observed in the $B\text{-III}^{**}_{RS}$ spectrum were enclosed in a block.

Assignments of Methyl Resonances of Ala(12) and Ala(29). Edman degradation was applied to B-III to assign the Ala(12) resonance, the P_2' residue of the first reactive site, Arg(10)–Arg(11), of the inhibitor. The Arg(10)–Arg(11) peptide bond was selectively cleaved by limited proteolysis so that the α-amino group of the P_1' residue was exposed and susceptible to Edman degradation. After Edman degradation, the P_1' resonance disappeared and the P_2' resonance shifted because of a positive charge on the newly generated amino group.

In des-V^1,R^{11},S^{39}-$B\text{-III}^{**}_{AN}$ obtained by a one-step Edman degradation of $B\text{-III}^{**}_{RS}$, the A_1 resonance shifted to lower field by 0.15 ppm, but the methyl resonance of the other alanyl residue, A_2, remained in the same position. Multiple-step Edman degradations on $B\text{-III}^{**}_{RS}$ are unsuitable since one of the disulfide loops is broken and a large conformational change may occur with the cleavage of the S–S loop (Norioka, 1984). Therefore, peanut inhibitor A-II was used to carry out more steps of Edman degradation. A-II has nine more residues in the N terminus than B-III, including an additional alanine located at position −8 (Figure 1). The methyl resonance of Ala(−8) in A-II was easily assigned by comparison of the COSY spectra of A-II and B-III. It appeared in the same position as the T_1 signal (i.e., overlapped with the T_1 resonance) in 1D spectra of A-II and $A\text{-II}^{**}_{RS}$.

In A-II, only the methyl resonance of Ala(−8) shifted about 0.14 ppm to lower field after one step of Edman degradation, and it disappeared after two steps. In $A\text{-II}^{**}_{RS}$, in addition to the methyl resonance of Ala(−8), only the A_1 signal also shifted approximately 0.14 ppm to lower field after one step of Edman degradation and disappeared after two steps (Table II). These results show that the A_1 resonance is Ala(12) and A_2 is Ala(29).

As shown in Table II, the T_3 signal was not observed after this procedure. Presumably it was shifted to higher field because of phenylthiocarbamylation on the ϵ-amino group of Lys(49) and therefore overlapped the resonances of the valines and a leucine.

Assignment of the Methyl Resonance of Thr(37). A carboxypeptidase was used to assign the P_1 and P_2 amino acid residues of the reactive sites in the enzymatically modified inhibitor.

Since Thr(37) is located at the P_2 position of the second reactive site of B-III, its methyl resonance was assigned by the proteolytic elimination of the P_1 amino acid residue by carboxypeptidase. When $B\text{-III}^{**}_{RS}$ was incubated with a catalytic amount of carboxypeptidase B for 24 h, Arg(10), Arg(38), Arg(60), and Ser(61) were released from the protein and des-R^{10},R^{38},R^{60},S^{61}-$B\text{-III}^{**}_{RS}$ was obtained. The release of Ser(61) may be due to a carboxypeptidase A contaminated in the commercial carboxypeptidase B.

The results are shown in Table I. The T_2 signal in des-R^{10},R^{38},R^{60},S^{61}-$B\text{-III}^{**}_{RS}$ at pH 3.3 appeared at about 0.05 ppm lower field than that of $B\text{-III}^{**}_{RS}$ and titrated from pH 3.3 to pH 4.5. This indicates that the T_2 signal is the resonance of the residue next to the C terminus in $B\text{-III}^{**}_{RS}$ and was assigned to Thr(37). The other signals, T_1, T_3, and T_4 (from two overlapped threonines), belonged to the four threonines that were located in the five S–S loops containing no cleavage sites.

Assignment of Aromatic Resonances. Inhibitor B-III contains four aromatic residues: one tyrosine at position 15; one histidine at position 26; two phenylalanines at positions 16 and 24.

The C-2 proton of His(26) was easily assigned from the value of its chemical shift, 8.63 ppm. The peak connected to this C-2 proton, by long-range coupling in the COSY spectrum and a nuclear Overhauser effect (NOE) in the NOESY spectrum, was assigned as the C-4 proton of His(26).

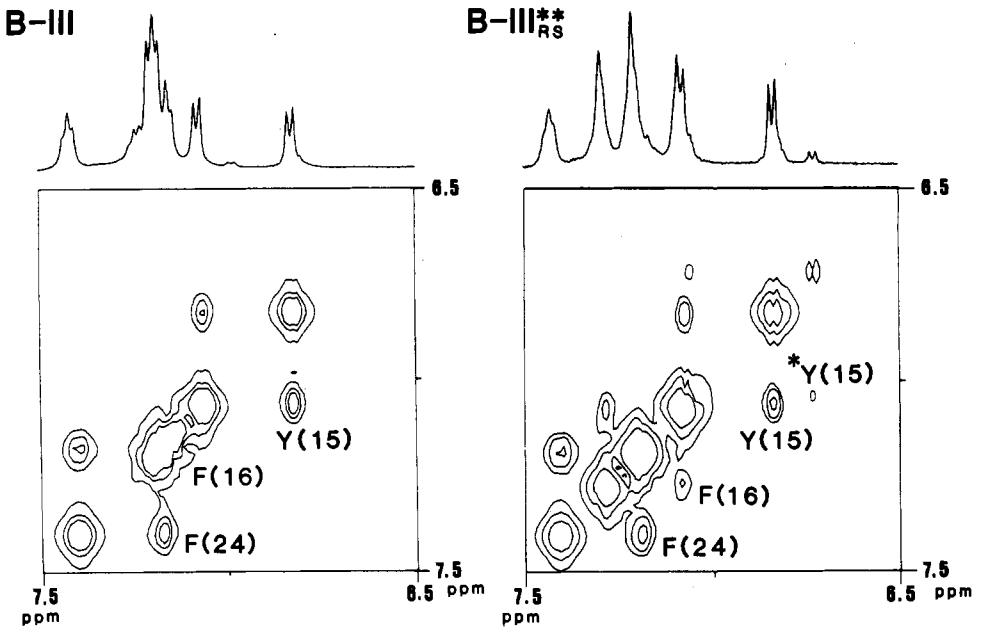

FIGURE 3: Aromatic regions of the COSY spectra of B-III and B-III$^{**}_{RS}$. Y*(15) comes from satellite resonances of Y(15) (see text).

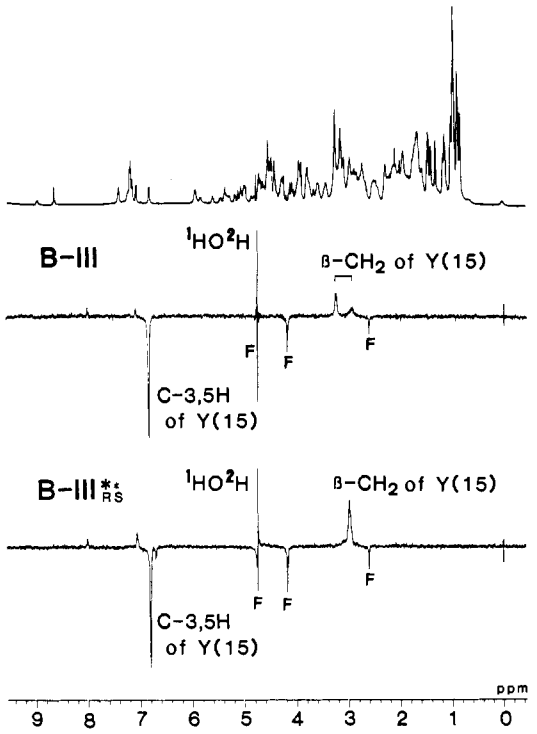

FIGURE 4: Photochemically induced dynamic nuclear polarization difference spectra of B-III and B-III$^{**}_{RS}$. The 0.4-mL sample solution contained 1.5 mM protein and 0.2 mM 3-*N*-(carboxymethyl)lumiflavin. Each spectrum was obtained by subtracting the dark spectrum from the light spectrum. F shows the resonance of the flavin dye.

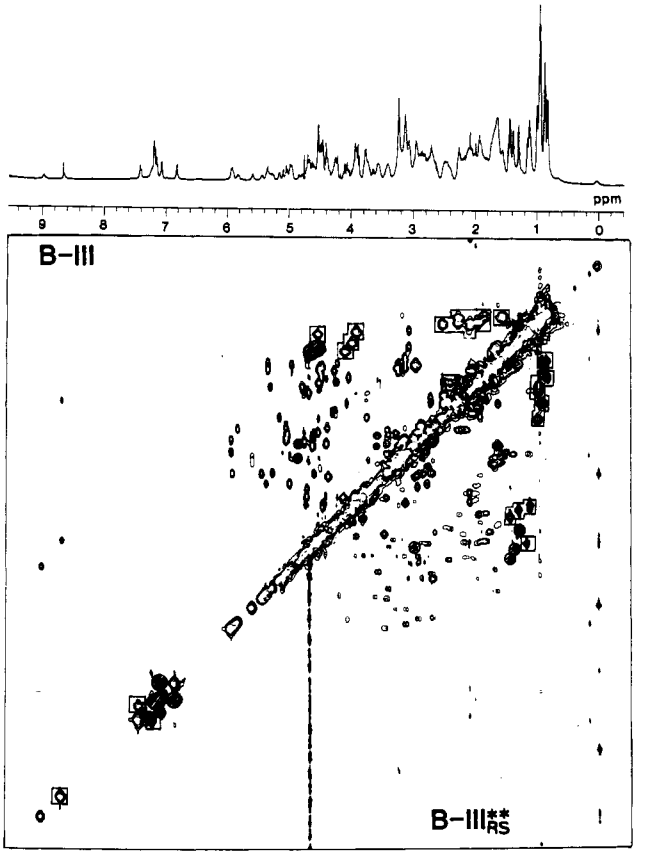

FIGURE 5: Combined COSY spectra of B-III and B-III$^{**}_{RS}$. The upper triangle of this contour plot was taken from the COSY spectrum of B-III and the lower triangle from that of B-III$^{**}_{RS}$. The 0.4-mL sample contained 8 mM protein and 10% acetic acid-d_4 in 100% ^2H_2O. The pH was 3.3, and the temperature was 40 °C. Among the cross peaks that were assigned or identified, the peaks enclosed in blocks appeared in the equivalent position in the both COSY spectra and the peaks enclosed in shaded circles appeared in the different position in the both spectra.

The typical chemical shift and the coupling pattern of AA'XX' doublets of tyrosine residue allowed the assignment of ring protons of Tyr(15) (Figure 3). The β-CH_2 protons of this residue were assigned from NOE connectivity with the ring protons observed in the NOESY spectrum and photochemically induced dynamic nuclear polarization data as will be mentioned below (Figure 4). The α-CH proton was assigned from spin coupling with the β-CH_2 observed in the COSY spectrum. An additional peak of Tyr(15) was observed in the COSY spectrum of B-III$^{**}_{RS}$ (Figure 3) which will be discussed below.

The other resonances in the aromatic region are phenylalanines. Limited proteolysis led to a change of the coupling pattern of the ring protons on one of two phenylalanines (Figure 5). This phenylalanine was shown to be located in vicinity of Tyr(15) because an NOE was observed between those aromatic ring protons in 1D NOE difference spectrum. Consequently, this phenylalanine was assigned to Phe(16) and

Table II: Proton Chemical Shifts of the Methyl Resonances in B-III, A-II, and Their Modified Derivatives by Edman Degradation

derivatives	chemical shifts of methyl resonances of Ala and Thr[a] (ppm)						
	A^{-8}	A_1	A_2	T_1	T_2	T_3	T_4
B-III		1.460	1.298	1.430	1.390	1.158	1.117
des-V^1-B-III[c]		1.457	1.268	1.422	1.390	NO[b]	1.112
$B\text{-}III^{**}_{RS}$		1.345	1.287	1.424	1.262	1.153	1.112
des-V^1,R^{11},S^{39}-$B\text{-}III^{**}_{AN}$[d]		1.496	1.280	1.424	1.255	NO	1.111
A-II	1.436	1.447	1.274	1.436	1.391	1.157	1.118
des-E^{-9}-A-II[e]	1.574	1.448	1.272	1.432	1.391	NO	1.114
des-E^{-9},A^{-8}-A-II[f]		1.449	1.273	1.432	1.391	NO	1.115
$A\text{-}II^{**}_{RS}$	1.430	1.357	1.280	1.430	1.260	1.151	1.111
des-E^{-9},R^{11},S^{39}-$A\text{-}II^{**}_{AN}$[g]	1.574	1.491	1.280	1.430	1.256	NO	1.109
des-E^{-9},A^{-8},R^{11},A^{12},S^{39},N^{40}-$A\text{-}II^{**}_{PP}$[h]			1.281	1.427	1.258	NO	1.108

[a] Shifts were measured relative to TSP, at 40 °C in ^2H_2O solution containing 5% acetic acid-d_4. The resonances of alanines and threonines were numbered from low to high field, respectively. A_1, A_2, and T_2 are assigned to the methyl resonances of Ala(12), Ala(29), and Thr(37), respectively, later. [b] Not observed. [c] B-III in which Val(1) is removed. [d] $B\text{-}III^{**}_{RS}$ in which Val(1), Arg(11), and Ser(39) are removed. [e] A-II in which Glu(−9) is removed. [f] A-II in which Glu(−9) and Ala(−8) are removed. [g] $A\text{-}II^{**}_{RS}$ in which Glu(−9), Arg(11), and Ser(39) are removed. [h] $A\text{-}II^{**}_{RS}$ in which Glu(−9), Ala(−8), Arg(11), Ala(12), Ser(39), and Asn(40) are removed.

the remaining one to Phe(24). The change in the coupling pattern observed for Phe(16) suggests that its side chain has greater mobility after limited proteolysis.

Photochemically Induced Dynamic Nuclear Polarization of Tyr(15). This technique shows whether aromatic residues of proteins are exposed to solvent or not. In general, a flavin dye is added to sample solution and excited to its triplet state by brief laser irradiation. The excited dye can form a radical pair with accessible tryptophan, tyrosine, and histidine residues, resulting in spin polarization accompanied by enhancement of signal intensity.

The difference spectra of B-III and $B\text{-}III^{**}_{RS}$ obtained by subtracting a dark spectrum from a light one are shown in Figure 4. The results indicated that the aromatic ring of Tyr(15) was highly exposed to the solvent in both inhibitors. The assignments of β-CH_2 resonances of Tyr(15) were confirmed by these spectra, and the resonances gave an AB pattern signal in B-III but a single peak in $B\text{-}III^{**}_{RS}$. As discussed above for Phe(16), the difference of the coupling patterns suggests that the side chain of Tyr(15) acquires a higher mobility after limited proteolysis. CIDNP of His is not detectable in such acidic conditions, pH 3.3 (Kaptein, 1982).

Comparison of 2D Spectra of B-III with Those of $B\text{-}III^{**}_{RS}$. Among 61 amino acid residues of B-III and $B\text{-}III^{**}_{RS}$, 8 residues have already been assigned and 9 more residues have been identified. Focusing on those 17 residues, the 2D spectra of B-III and $B\text{-}III^{**}_{RS}$ were compared. The profile of the cross peaks is good for detecting the changes of protein conformation (Figures 5 and 6). Some differences in coupling patterns and chemical shifts of COSY and NOE profiles of NOESY spectra were detected for Ala(12), Tyr(15), Phe(16), and Thr(37). It should be noted that all of these residues were located in the S–S loop containing the reactive sites, the P_2', P_5', and P_6' positions of the first reactive site and the P_2 position of the second reactive site, respectively. There were no differences for Leu(7), Phe(24), His(26), Ala(29), five valines, and four threonines, which are located in the five S–S loops containing no reactive site. No large changes were observed in the remaining two-thirds of the cross peaks, which have not been assigned or identified.

These facts clearly show that the conformational change, induced by limited proteolysis, is not so large and is localized in the two S–S loops where peptide bonds are cleaved.

Temperature and pH Dependences. The differences between the B-III and $B\text{-}III^{**}_{RS}$ conformations were investigated further by measuring the temperature and pH dependences of their spectra. In particular, chemical shifts were followed as a function of either temperature or pH, for the resolved peaks for the ring protons of Tyr(15), the four methyl peaks of Ala(12), Ala(29), Thr(37), and threonine T_1 (Figures 7 and 8).

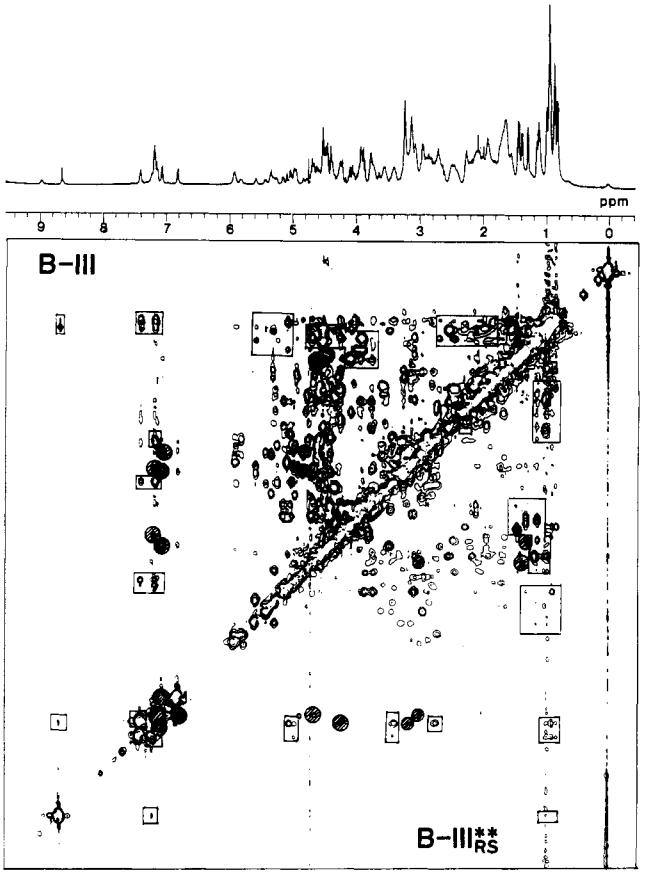

FIGURE 6: Combined NOESY spectra of B-III and $B\text{-}III^{**}_{RS}$. The upper triangle of this contour plot was taken from the NOESY spectrum of B-III and the lower triangle from that of $B\text{-}III^{**}_{RS}$. These spectra were obtained under the same conditions described in Figure 5. Among the cross peaks that were assigned or identified, the peaks enclosed in blocks appeared in the equivalent position in the both NOESY spectra, and the peaks enclosed in shaded circles appeared in the different position in both spectra.

The temperature and pH dependencies of the resonance of Ala(12) were different in B-III and $B\text{-}III^{**}_{RS}$, but Thr(37) and Tyr(15) are slightly sensitive to either the variation of temperature or pH. Ala(29) and threonine T_1 show no chemical shift changes with either temperature or pH changes. These results confirmed results from the 2D experiments where

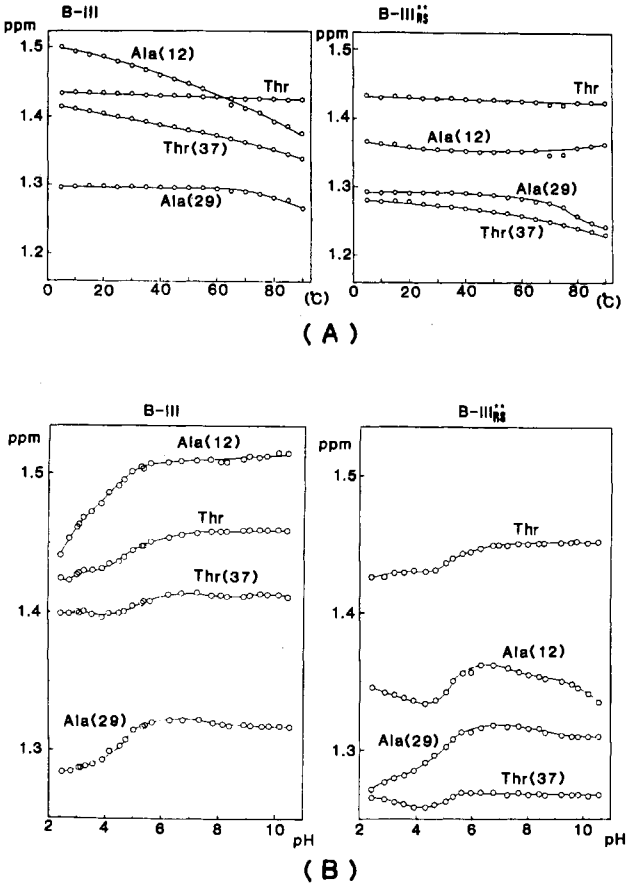

FIGURE 7: (A) Temperature dependence of the methyl resonances of B-III and B-III$^{**}_{RS}$ in ^2H_2O solution containing 5% acetic acid-d_4. Thr indicates the T_1 signal of threonine. (B) pH dependence of the methyl resonances of B-III and B-III$^{**}_{RS}$ at 40 °C. Thr indicates the T_1 signal of threonine.

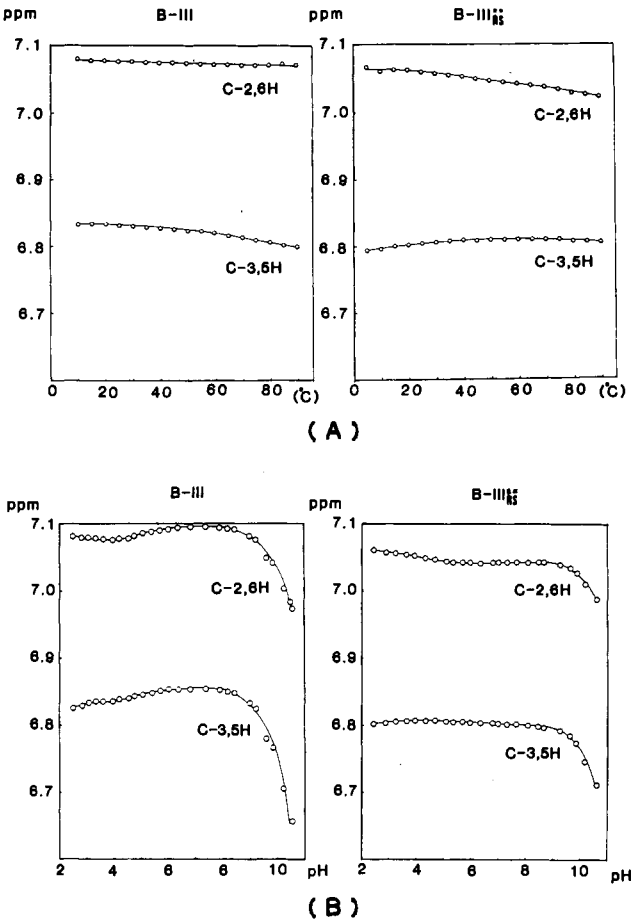

FIGURE 8: (A) Temperature dependence of the ring protons of Tyr(15) of B-III and B-III$^{**}_{RS}$ in ^2H_2O solution containing 5% acetic acid-d_4. (B) pH dependence of the ring protons of Tyr(15) of B-III and B-III$^{**}_{RS}$ at 40 °C.

Ala(29) and a threonine of T_1 did not show conformational change and the changes were localized in the two S–S containing Ala(12), Tyr(15), and Thr(37).

Molecular Conformation and Inhibitory Activity of B-III. The conformational differences induced by the cleavage of peptide bonds in protease inhibitors have been investigated by NMR measurements. Similarly, results for basic pancreatic trypsin inhibitor (BPTI) (Wagner et al., 1979) and *Streptomyces* subtilisin inhibitor (SSI) (Akasaka et al., 1983) have shown that conformational changes occur only near the modified sites. B-III showed the same behavior as these inhibitors. Cleavage of the scissile peptide bonds of BPTI and SSI reduced their thermal stabilities. However, both B-III and B-III$^{**}_{RS}$ were so stable that there is no evidence of denaturation up to 90 °C.

The resonance positions of Ala(29) and threonine T_1, which did not show any chemical shift with cleavage, were constant throughout the temperature range. This suggests that these residues are located in a conformationally stable region of the protein. On the other hand, the chemical shifts of Ala(12) and Thr(37) of B-III showed a monotonic change with increasing temperature, and their values extrapolated to 100 °C nearly correspond to chemical shifts of Ala(29) and T_1 of B-III$^{**}_{RS}$ (which show almost no temperature dependency). These temperature dependencies reveal that the two disulfide loops containing the cleaved sites have flexibilities in B-III. Temperature and pH changes increase the motion of these chains. The high thermal stability of the remaining part, which consists of five S–S loops and will be called a core region, may result from the rigid structure caused by seven S–S bonds.

The stability of the core region may also be explained by β-sheet structure. The presence of a β-sheet structure in B-III is suggested from the following observations: more that 15 protons found in the region between 5 and 6 ppm are downfield-shifted C_α protons, and NOEs were detected between pairs of C_α protons. Moreover, there were at least two amide protons in B-III that were not easily exchanged with deuterium in ^2H_2O solution at pH 3.3. They are spin coupled with the low-field shifted C_α protons in the COSY spectrum. Such phenomena have been observed for protons in residues in β-sheet structures (Dubs et al., 1979; Inagaki et al., 1982). The structure in which the flexible reactive site is attached to the rigid core region might be essential to the inhibitory activity of B-III.

Izumiya's group synthesized the cyclic nona- and hexadecapeptides, which correspond to the outermost loop and to the double loop of BBI, respectively, and showed that the K_i values of their complexes with trypsin are 7.5×10^{-7} and 1.0×10^{-7} M, respectively, compared to a value of 3.7×10^{-9} M for BBI. This nonapeptide lost its inhibitory activity by proteolysis when it was incubated with trypsin for a long time (Nishino et al., 1977; Nishino & Izumiya, 1982). Norioka reported that when one of the S–S loops is split by the cleavage of the Cys(2)–Cys(3) peptide bond, the inhibitory activity of B-III is reduced to one-tenth that of the native inhibitor. Furthermore the splitting of the S–S loop by cleavage of the Cys(3)–Asn(4) bond lowered the activity further by another factor of 10 (Norioka, 1984). It is also considered from all these results that the rigidity of the core region is necessary for its activity.

There were additional signals in the COSY spectrum of

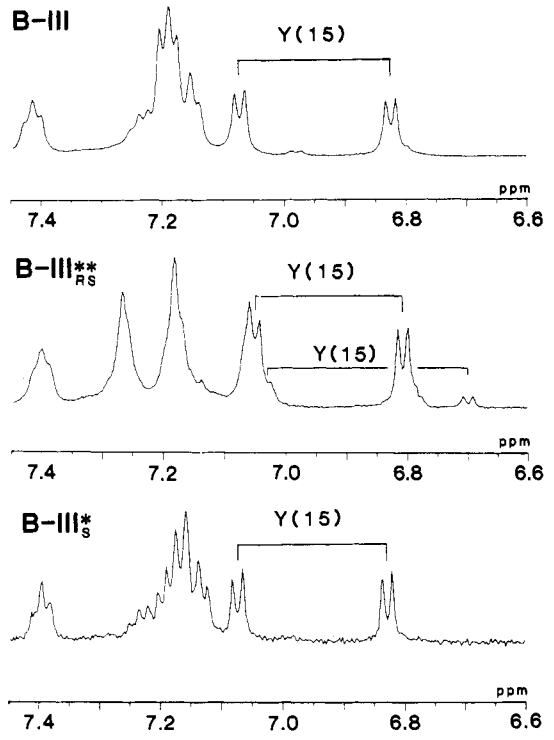

FIGURE 9: Aromatic region of B-III, B-III$^{**}_{RS}$, and B-III$^{*}_{S}$. Each protein was dissolved in 0.4 mL of ^2H_2O containing 5% acetic acid-d_4, and its spectrum was measured at 40 °C. The sample concentration was 3.5 mM in B-III and B-III$^{**}_{RS}$ and 1.2 mM in B-III$^{*}_{S}$.

B-III$^{**}_{RS}$ that were assigned to the satellite signals of Ala(12) and Tyr(15), because of their characteristic patterns. The chemical shift and the coupling pattern of the signals of Ala(29) did not change with the cleavage of two peptide bonds, and therefore the assignment of the satellite to this residue was excluded.

Furthermore, the satellite signal of Tyr(15) disappeared in the spectrum of B-III$^{*}_{S}$ that was obtained by the regeneration of the cleaved bond at Arg(10)–Arg(11) with chymotrypsin (Figure 9). The occurrence of these satellites is attributed to slow cis–trans isomerization about peptide bonds involving Pro(13) and Pro(14). The exchange rate of trans and cis conformers is so slow and shows separate resonances in NMR (Deber et al., 1970). Thus, these satellite signals show that these proline residues have gained rotational freedom after the cleavage of the Arg(10)–Arg(11) bond. Other support for increased motion is the change in coupling patterns of the β-CH_2 protons and ring protons of Tyr(15) and Phe(16), respectively.

B-III is characterized by its inhibitory activity for chymotrypsin (Norioka & Ikenaka, 1984). The first reactive site, Arg(10)–Arg(11), inhibits both trypsin and chymotrypsin. Because chymotrypsin preferentially hydrolyzed peptide bonds in which the carboxyl group is contributed by the aromatic amino acids such as phenylalanine, tyrosine, or tryptophan, the peptide bond of this site is difficult to cleave by chymotrypsin. Thus, the antichymotryptic activity can be understood if it is assumed that chymotrypsin binds firmly to B-III at its reactive site, which is located in some charateristic three-dimensional structure recognized by the active site of the enzyme. The destruction of this special conformation, as demonstrated by the NMR experiments, explains why the B-III$^{**}_{RS}$ has little inhibitory activity for chymotrypsin.

Conclusion

We have applied Edman degradation and carboxypeptidase treatment and demonstrated how to use them for assigning NMR resonances of residues around the reactive site of the stable protease inhibitors, such as B-III. Using these chemical modifications and two-dimensional NMR techniques in addition to conventional NMR techniques, we have assigned and/or identified 17 residues of peanut protease inhibitor B-III and its modified form B-III$^{**}_{RS}$.

The molecule appears to contain three domains: two flexible S–S loops, each containing a reactive site, and one rigid core region stabilized by seven S–S bonds. When the molecule is cleaved by trypsin at either reactive site, local conformational changes occur independently on each of the S–S loops.

Registry No. Proteinase inhibitor, 37205-61-1; trypsin inhibitor, 9035-81-8; chymotrypsin, 9004-07-3.

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