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A New Family of Serine Protease Inhibitors (Bombyx Family) as Established from the Unique Topological Relation between the Positions of Disulfide Bridges and Reactive Site

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The positions of the reactive site and the disulfide bridges in fungal protease inhibitor F (FPI-F) from silkworm (*Bombyx mori*), which has a unique amino acid sequence and inhibitory specificity, were investigated. At pH 3.0, subtilisin BPN′, which is one of target proteases of this inhibitor, specifically cleaved the peptide bond of the inhibitor at Thr(29)-Val(30). The cleaved bond was regenerated by subtilisin BPN′ at pH 8.0. These results indicate that the Thr(29)-Val(30) bond of the inhibitor is the reactive site. The locations of disulfide bridges were determined to be Cys(3)-Cys(35), Cys(14)-Cys(27), Cys(18)-Cys(55), and Cys(37)-Cys(49). Based on the positions of the reactive site and the disulfide bridges, FPI-F is considered to be a member of a new family of serine protease inhibitors. We propose the designation Bombyx family for the new inhibitor family of which FPI-F is a member.

Key words: Bombyx family inhibitor, *Bombyx mori*, disulfide bridge, fungal protease inhibitor, reactive site.

The protease inhibitory activity in larval hemolymph of silkworm (*Bombyx mori*) was first reported by Morita and Kikkawa (*1*). Since then, the protease inhibitors in hemolymph of silkworm have been extensively studied, and they include inhibitors of various proteases such as chymotrypsin, trypsin, fungal proteases, and so on (*2-12*). Among them, fungal protease inhibitor F (FPI-F) has a unique amino acid sequence and inhibitory specificity against fungal proteases (*13, 14*). The inhibitor is remarkably stable at lower pH and at higher temperature. It consists of 55 amino acids with 8 cysteine residues, which are supposed to form 4 disulfide bridges. The amino acid sequence has no significant homology with that of any other known protease inhibitor (*14*), suggesting that FPI-F belongs to a new family of serine protease inhibitors. However, the locations of the reactive site and the disulfide bridges of FPI-F, which are essential for the classification of serine protease inhibitors, have not been established yet. The present paper describes the determination of the positions of the reactive site and the disulfide bridges of FPI-F.

MATERIALS AND METHODS

Materials—FPI-F was purified in our laboratory according to the method reported previously (*14*). Subtilisin BPN′, thermolysin and methanesulfonic acid (4 M) containing 0.2% 3-(2-aminoethyl)indole were from Wako Pure Chemical Industries (Osaka). TPCK-trypsin from Sigma (St. Louis, MO, USA) and Cosmosil $5C_{18}$ (4.6×150 mm) from Nacalai Tesque (Kyoto) were used. Semi-alkaline protease of *Aspergillus melleus* (twice crystallized) was a product of Amano Chemicals (Nagoya). All other chemicals were of the finest grade commercially available.

Amino Acid Analysis—Samples were hydrolyzed at 110°C for 24 h in sealed and evacuated glass tubes with 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole. Amino acid analyses were done with an amino acid analyzer (Hitachi, model 835).

Sequence Analysis—The amino acid sequence analyses were performed with an automatic gas phase sequencer (Shimadzu, model PPSQ-10).

Reversed-Phase HPLC—A column of Cosmosil $5C_{18}$ (4.6×150 mm) was used for HPLC. For purification of modified FPI-F (M-FPI-F), regenerated FPI-F, and RCm-fragments derived from M-FPI-F, the column was eluted with an 80-min linear gradient from 0 to 80% acetonitrile in 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 ml/min and inhibitors were monitored at 230 nm. For separation of fragments containing cystine, a 40-min gradient from 0 to 40% acetonitrile in 0.1% TFA was used at a flow rate of 1 ml/min and the peptides were monitored at 230 nm. For the rechromatography of fragments containing cystine, 10 mM ammonium acetate buffer (pH 5.6) instead of 0.1% TFA was used, with a linear gradient from 0 to 40% acetonitrile over 50 min.

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Abbreviations: FPI-F, fungal protease inhibitor F of silkworm; M-FPI-F, modified FPI-F in which a peptide bond of the reactive site is cleaved; PTH, phenylthiohydantoin; RCm, reduced and *S*-carboxymethylated; TFA, trifluoroacetic acid.

Limited Proteolysis of FPI-F with Subtilisin BPN'—FPI-F (30 nmol) was dissolved in 300 μl of 50 mM citrate buffer (pH 3.0) and subtilisin BPN' (4 nmol) was added. After incubation for 2 days at room temperature, further subtilisin BPN' (4 nmol) was added and the mixture was kept for another 2 days. Then M-FPI-F was purified by reversed-phase HPLC.

Reduction and S-Carboxymethylation—The M-FPI-F was reduced and *S*-carboxymethylated by the method of Crestfield *et al.* (*15*). The reduced and *S*-carboxymethylated (RCm) fragments obtained were purified by reversed-phase HPLC.

Regeneration of M-FPI-F to FPI-F—The M-FPI-F was converted into FPI-F according to the method of Ozawa and Laskowski, Jr. (*16*). The M-FPI-F (5.6 nmol) was mixed with subtilisin BPN' (7 nmol) in 400 μl of 0.27 M Tris-HCl buffer (pH 8.0) and the mixture was incubated at 25°C for 15 min to form the enzyme-inhibitor complex. The mixture was then adjusted to pH 1.5 at 0°C by addition of 40 μl of 10% TFA to decompose the enzyme-inhibitor complex. After standing at 0°C for 3 min, the mixture was subjected to reversed-phase HPLC.

Ellman Reaction—For measurement of the amounts of sulfhydryl groups, FPI-F (5-25 nmol) was dissolved in 1 ml of 0.1 M Tris-HCl at pH 8.0 containing 0.01 M EDTA and 6 M guanidine-HCl. To the FPI-F solution was added 40 μl of freshly prepared 5,5'-dithiobis(2-nitrobenzoic acid) solution (0.01 M in 0.05 M sodium phosphate buffer, pH 7.0). Then the change of absorbance at 412 nm was recorded (*17*).

Determination of Disulfide Bridges—To cleave the Asp(20)-Pro(21) bond of M-FPI-F, M-FPI-F (20 nmol) was dissolved in 100 μl of 70% formic acid and kept at 40°C for 1 week. After lyophilization, the sample was digested with thermolysin (0.3 nmol) at 30°C for 22 h in 100 mM ammonium acetate buffer (pH 6.5) containing 0.025 M $CaCl_2$ and 6 M urea. After thermolysin digestion, the concentration of urea in the reaction mixture was reduced to 2 M by addition of 100 mM ammonium acetate buffer (pH 6.5) containing 0.025 M $CaCl_2$. TPCK-trypsin (0.3 nmol) was added to the mixture. The whole was further incubated at 30°C for 20 h and the fragments were separated by reversed-phase HPLC. The structures of the fragments containing cystine were confirmed by the results of Edman degradation, amino acid analysis, and mass spectrometry (Perkin Elmer SCIEX, API-III).

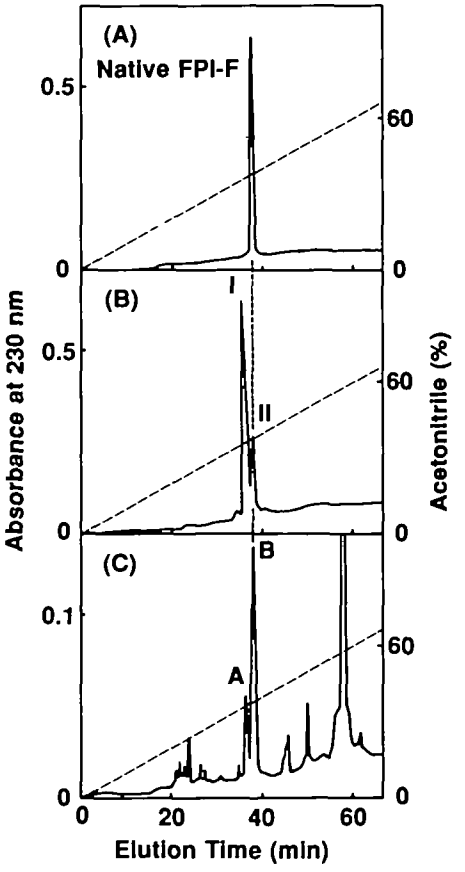

Fig. 1. **Limited proteolysis and regeneration of the reactive-site peptide bond of FPI-F.** Native and modified FPI-F were separated by reversed-phase HPLC. The samples were put on a Cosmosil $5C_{18}$ column (4.6×150 mm) equilibrated with 0.1% TFA. The column was eluted with a linear gradient of acetonitrile from 0 to 80% for 80 min. Proteins were monitored at 230 nm. (A), native FPI-F; (B), FPI-F after limited hydrolysis with subtilisin BPN'; (C), regenerated FPI-F.

RESULTS

Determination of the Reactive Site—Since FPI-F strongly inhibits subtilisin BPN', subtilisin BPN' was used for the determination of the reactive site of FPI-F. FPI-F was digested with subtilisin BPN' at pH 3.0 and was subjected to reversed-phase HPLC as illustrated in Fig. 1B. Comparing the retention time with that of native FPI-F (Fig. 1A), it was supposed that peaks I and II were modified FPI-F (M-FPI-F), in which reactive-site peptide bond was cleaved, and native FPI-F, respectively. To determine the cleavage site of FPI-F by subtilisin BPN', M-FPI-F (peak I) and FPI-F (peak II) were sequenced. The sequence analysis of M-FPI-F (peak I) showed a sequence, Val-Ile-Arg-Glu-Thr, corresponding to positions 30 to 34 of FPI-F in addition to the sequence from the amino terminus. That of FPI-F (peak II) showed a single sequence that coincided with the N-terminal sequence of FPI-F. These findings indicated that the Thr(29)-Val(30) bond of FPI-F was

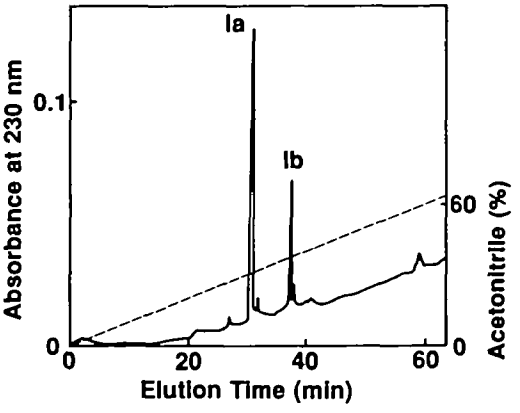

Fig. 2. **Reversed-phase HPLC of RCm-M-FPI-F.** RCm-M-FPI-F was put on a Cosmosil $5C_{18}$ column (4.6×150 mm) equilibrated with 0.1% TFA. The column was eluted with a linear gradient of acetonitrile from 0 to 80% for 80 min. Peptides were monitored at 230 nm.

specifically cleaved by subtilisin BPN'.

Since the two peptides formed by the cleavage of the peptide bond at the reactive site were thought to be linked by disulfide bridges, M-FPI-F was reduced and *S*-carboxymethylated. The RCm-fragments were separated by reversed-phase HPLC and two peaks, Ia and Ib, were obtained (Fig. 2). Judging from the amino acid compositions (Table I) and the amino acid sequences, Ia and Ib were determined to be peptides, Leu(1)-Thr(29) and Val(30)-Cys(55), respectively. As it is well known that a valyl-isoleucyl bond is resistant to hydrolysis, lower values of valine and isoleucine in the amino acid composition of Ib (Table I) might be due to the Val(30)-Ile(31) bond. Thus, it was confirmed that subtilisin BPN' specifically hydrolyzed the Thr(29)-Val(30) bond of FPI-F and that the two peptides formed were linked by disulfide bridges.

For further confirmation of the position of the reactive site, the peptide bond between Thr(29) and Val(30) of M-FPI-F was regenerated. M-FPI-F was incubated with a

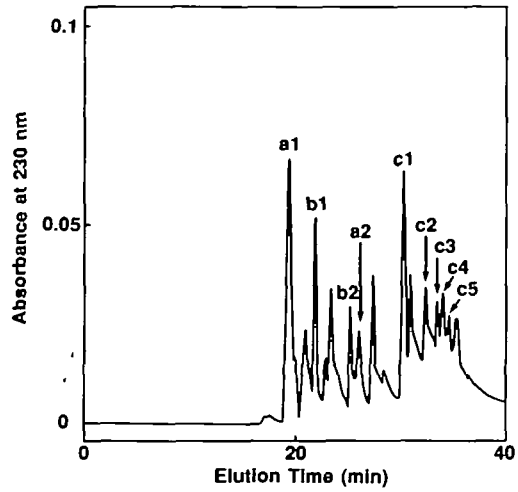

Fig. 3. **Reversed-phase HPLC of cystine-containing fragments.** M-FPI-F treated with 70% formic acid was digested with thermolysin and trypsin. Then, the mixture was put on a Cosmosil $5C_{18}$ column (4.6×150 mm) equilibrated with 0.1% TFA. The column was eluted with a linear gradient of acetonitrile from 0 to 40% for 40 min. Fragments were monitored at 230 nm.

TABLE I. **Amino acid compositions of RCm-fragments of M-FPI-F, Ia and Ib.**

	Ia	L_1-T_{29}[a]	Ib	V_{30}-C_{55}[b]
Cm-Cys	3.6 (4)	4	3.7 (4)	4
Asp	4.6 (5)	5	2.4 (2)	2
Thr	1.9 (2)	2	0.9 (1)	1
Ser	2.7 (3)	3	3.0 (3)	3
Glu	3.3 (3)	3	3.2 (3)	3
Pro	3.7 (4)	4	1.2 (1)	1
Gly	1.2 (1)	1	1.4 (1)	1
Val	1.0 (1)	1	2.3 (2)	3
Ile	0.9 (1)	1	2.2 (2)	3
Leu	1.0 (1)	1	0.8 (1)	1
Tyr	0.9 (1)	1	–	0
Phe	–	0	0.8 (1)	1
Lys	0.9 (1)	1	0.9 (1)	1
His	–	0	0.9 (1)	1
Arg	1.8 (2)	2	1.0 (1)	1
Total	29	29	26	26
Yield (%)	38		38	

[a]Indicates the amino acid composition of the peptide Leu(1)-Thr(29). [b]Indicates the amino acid composition of the peptide Val(30)-Cys(55).

TABLE III. **The results of amino acid sequence analyses of fragments a1 and a2.** Numbers in parentheses indicate the yield of PTH-amino acids (%).

Cycle	PTH-amino acids	
	a1	a2
1	Thr (12.1)	Thr (13.3)
	Ile (44.1)	Ile (47.7)
2	Ser (6.7)	Ser (6.0)
3	Asn (30.1)	Asn (37.7)
	Gln (28.2)	Gln (28.2)
4	Asp (10.3)	Asp (16.9)
	$(Cys)_2$	$(Cys)_2$
5	nd[a]	Pro (46.6)
6	nd	Tyr (29.4)
7	nd	Gly (23.7)
8	nd	Gln (19.6)
9	nd	Asn (12.1)

[a]nd, not detected.

TABLE II. **Amino acid compositions of cystine-containing fragments.** The numbers of residues in parentheses are from the established sequences.

Amino acid	a1	a2	b1	b2	c1	c2	c3	c4	c5
Asp	2.1 (2)	3.3 (3)	2.0 (2)	2.3 (2)		2.0 (2)	2.4 (2)	1.9 (2)	2.3 (2)
Thr	1.0 (1)	1.0 (1)		0.8 (1)	1.0 (1)	1.0 (1)	1.3 (1)	1.3 (1)	1.3 (1)
Ser	1.0 (1)	0.7 (1)	2.0 (2)	2.1 (2)		1.6 (2)	1.7 (2)	2.1 (2)	2.3 (2)
Glu	1.2 (1)	1.7 (2)		1.3 (1)	2.9 (3)	3.1 (3)	3.4 (3)	3.3 (3)	3.4 (3)
Pro		0.7 (1)	1.7 (2)	2.6 (3)	2.3 (2)	2.1 (2)	2.4 (2)	2.2 (2)	2.3 (2)
Gly		1.1 (1)		1.3 (1)	1.2 (1)	1.1 (1)	1.1 (1)	1.3 (1)	1.2 (1)
1/2Cys	1.7 (2)	1.6 (2)	2.1 (2)	1.9 (2)	3.5 (4)	3.4 (4)	3.5 (4)	3.5 (4)	3.4 (4)
Val					1.2 (1)	1.3 (1)	1.8 (2)	1.3 (1)	1.8 (2)
Ile	0.9 (1)	1.0 (1)		1.1 (1)	0.9 (1)	0.8 (1)	1.1 (1)	1.1 (1)	0.9 (1)
Leu					1.1 (1)	1.2 (1)	1.3 (1)	1.8 (2)	2.2 (2)
Tyr		1.2 (1)		1.0 (1)					
Phe						1.3 (1)	0.7 (1)	0.9 (1)	0.8 (1)
Lys					1.7 (2)	1.7 (2)	2.0 (2)	2.0 (2)	2.0 (2)
His					0.9 (1)	0.9 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Arg			0.9 (1)	0.7 (1)					
Total residues	8	13	9	15	17	22	23	23	24
Yield (%)	32	6	26	10	13	15	8	8	7

slight excess of subtilisin BPN′ at pH 8.0. Then, the mixture was acidified with TFA to dissociate the inhibitor-enzyme complex and subjected to reversed-phase HPLC (Fig. 1C). The retention times of peaks A and B, both having the same amino acid composition as that of FPI-F, were identical to those of M-FPI-F and native FPI-F, respectively. Based on the HPLC result of an experiment using the reaction mixture without inhibitor, other peaks in Fig. 1C might be caused by autolysis of subtilisin BPN′. Peak B gave a single N-terminal amino acid sequence, Leu-Gln-Xaa-Pro-Lys, which was identical with that of native FPI-F (Xaa indicates that PTH-amino acid was not detected because of cystine). The result indicated that the peak B was FPI-F formed from M-FPI-F, and the cleaved Thr(29)-Val(30) peptide bond was regenerated by subtilisin BPN′. The yield of resynthesized FPI-F was 68%. Thus, the position of the reactive site of FPI-F was confirmed to be the Thr(29)-Val(30) bond. The same result was obtained when semi-alkaline protease from *Aspergillus melleus* was used as the target enzyme.

Location of Disulfide Bridges—The negative result of the Ellman reaction showed that FPI-F had no free sulfhydryl group. Thus, it was supposed that FPI-F contained four disulfide bridges. M-FPI-F (20 nmol) was incubated at 40°C for 1 week in 70% formic acid to cleave the Asp(20)-Pro(21) bond. The result of amino acid sequence analysis showed that 79% of the Asp(20)-Pro(21) bond of M-FPI-F was cleaved by formic acid without any other bond cleavage. This sample was further digested with thermolysin and TPCK-trypsin and was subjected to reversed-phase HPLC to separate the fragments. The result is illustrated in Fig. 3. Cystine-containing fragments were purified by rechromatography. The amino acid compositions of the fragments obtained are listed in Table II.

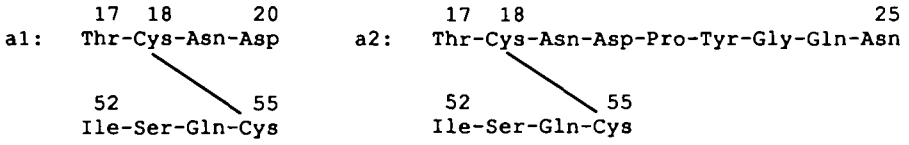

Fig. 4. **Proposed structures of fragments a1 and a2.**

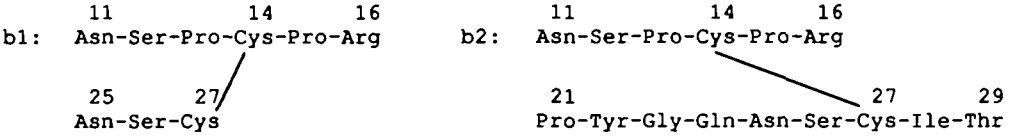

Fig. 5. **Proposed structures of fragments b1 and b2.**

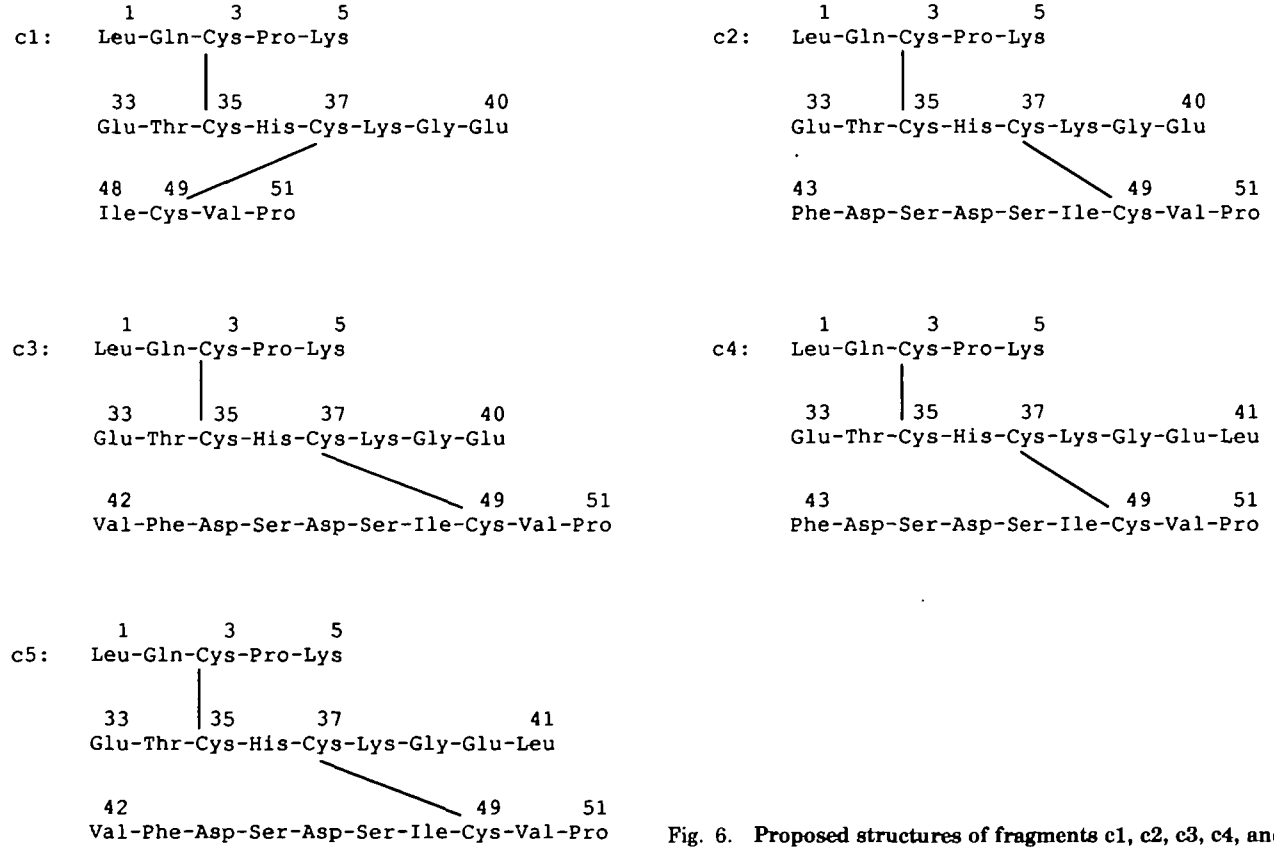

Fig. 6. **Proposed structures of fragments c1, c2, c3, c4, and c5.**

TABLE IV. **The results of amino acid sequence analyses of fragments b1 and b2.** Numbers in parentheses indicate the yield of PTH-amino acids (%).

Cycle	PTH-amino acids	
	b1	b2
1	Asn (97.8)	Asn (45.2)
		Pro (45.1)
2	Ser (72.8)	Ser (9.9)
		Tyr (46.8)
3	Pro (87.6)	Pro (37.0)
		Gly (38.5)
4	$(Cys)_2$	Gln (34.6)
5	Pro (44.1)	Pro (31.7)
		Asn (31.5)
6	Arg (55.8)	Arg (4.4)
		Ser (6.1)
7	nd[a]	$(Cys)_2$
8	nd	Ile (28.5)
9	nd	Thr (2.2)

[a]nd, not detected.

TABLE V. **The results of amino acid sequence analyses of fragments c1, c2, c3, c4, and c5.** Numbers in parentheses indicate the yield of PTH-amino acids (%).

Cycle	PTH-amino acids				
	c1	c2	c3	c4	c5
1	Leu (97.8)	Leu (76.2)	Leu (73.5)	Leu (70.0)	Leu (44.5)
	Glu (82.0)	Glu (30.9)	Glu (65.9)	Glu (70.0)	Glu (49.8)
	Ile (69.5)	Phe (48.7)	Val (45.6)	Phe (33.3)	Val (31.1)
2	Gln (72.8)	Gln (19.6)	Gln (45.2)	Gln (49.5)	Gln (28.9)
	Thr (44.5)	Thr (9.3)	Thr (18.7)	Thr (19.0)	Thr (13.5)
		Asp (18.3)	Phe (40.6)	Asp (14.4)	Phe (27.1)
3	$(Cys)_2$	$(Cys)_2$	$(Cys)_2$	$(Cys)_2$	$(Cys)_2$
	Val (45.3)	Ser (6.1)	Asp (16.2)	Ser (4.4)	Asp (10.8)
4	Pro (84.7)	Pro (21.1)	Pro (47.0)	Pro (53.1)	Pro (27.0)
	His (19.7)	His (5.5)	His (11.0)	His (13.0)	His (6.3)
		Asp (21.2)	Ser (4.2)	Asp (15.2)	Ser (2.4)
5	Lys (44.1)	Lys (10.1)	Lys (23.7)	Lys (33.8)	Lys (16.5)
	$(Cys)_2$	Ser (4.1)	Asp (13.5)	Ser (3.3)	Asp (8.9)
6	Lys (55.8)	Lys (17.8)	Lys (34.4)	Lys (36.1)	Lys (20.9)
		Ile (17.3)	Ser (3.3)	Ile (11.0)	Ser (2.2)
7	Gly (29.2)	Gly (10.6)	Gly (18.8)	Gly (20.2)	Gly (11.7)
		$(Cys)_2$	Ile (13.0)	$(Cys)_2$	Ile (9.2)
8	Glu (14.4)	Glu (2.6)	Glu (5.4)	Glu (9.5)	Glu (6.2)
		Val (10.9)	$(Cys)_2$	Val (7.0)	$(Cys)_2$
9	nd[a]	Pro (5.9)	Val (8.5)	Leu (3.2)	Leu (2.0)
				Pro (3.2)	Val (7.5)
10	nd	nd	Pro (3.9)	nd	Pro (1.9)

[a]nd, not detected.

On Edman degradation of fragment a1, two PTH-amino acids were detected at each cycle, except for the second and the fourth cycles and PTH-cystine was detected at the fourth cycle (Table III). From the results of sequencing and amino acid analysis, the position of the disulfide bridge at Cys(18)-Cys(55) was confirmed, as shown in Fig. 4. This was also confirmed by the analysis of fragment a2 (Table III and Fig. 4).

On Edman degradation of fragment b1, a single PTH-amino acid was detected at each cycle (Table IV). PTH-cystine was detected at the fourth cycle. From the amino acid composition (Table II) and sequence, the structure of fragment b1 was speculated to be as illustrated in Fig. 5. The result indicated that fragment b1 was composed of two peptides linked by a disulfide bridge at Cys(14)-Cys(27), as shown in Fig. 5. This result was also supported by the amino acid sequence of fragment b2 (Fig. 5).

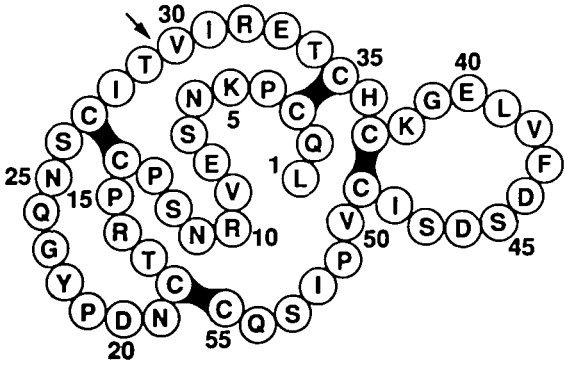

Fig. 7. **Proposed covalent structure of FPI-F.** The arrow indicates the reactive site.

On Edman degradation of c1, which contained four cysteine residues, three PTH-amino acids were identified at the first cycle and PTH-cystine was detected at the third and the fifth cycles (Table V). The result of Edman degradation indicated that three peptides, Leu(1)-Lys(5), Glu(33)-Glu(40), and Ile(48)-Pro(51) were linked by two disulfide bridges, as shown in Fig. 6. The mass number of 1920.01 of fragment c1 obtained by mass spectrometry coincided with the calculated value of 1920.3. This result supported the view that fragment c1 had two disulfide bridges, as shown in Fig. 6. However, we could not determine the positions of the disulfide bridges by the sequencing analysis of fragment c1. Fortunately, PTH-cystine was detected at the third and the seventh cycles of the Edman degradation of c2. This result indicated that disulfide bridges were formed at Cys(3)-Cys(35) and Cys(37)-Cys(49), as shown in Fig. 6. If disulfide bridges are formed at Cys(3)-Cys(37) and Cys(35)-Cys(49), PTH-cystine ought to be detected at the fifth and the seventh cycles. The same results were obtained in the sequence analyses of c3, c4, and c5 (Table V). Thus, we could determine the locations of all disulfide bridges as Cys(18)-Cys(55), Cys(14)-Cys(27), Cys(3)-Cys(35), and Cys(37)-Cys(49), as shown in Fig. 7.

DISCUSSION

The serine protease inhibitors have been classified into 10 or more families based on the homologies in amino acid sequences and in the topological relation between the positions of the disulfide bridges and the reactive site (*18*).

Owing to the unique character of the amino acid sequence of FPI-F (*14*), this inhibitor was supposed to belong to a new family of serine protease inhibitors. To confirm this, it is necessary to know not only the amino acid sequence, but also the positions of the reactive site and the disulfide bridges.

The determination of the reactive site of the serine protease inhibitor was first done in the case of soybean trypsin inhibitor (Kunitz) (*16*), by means of a two-step procedure of limited proteolysis at the reactive site with trypsin at acidic pH, followed by regeneration of the scissile bond by the formation of the complex with trypsin at neutral pH. This procedure is a standard method for the

determination of the reactive site of serine protease inhibitors.

The cleavage of the reactive-site peptide bond of FPI-F with subtilisin BPN′ was successful at pH 3.0. The new peak on reversed-phase HPLC (Fig. 1B) was identified as M-FPI-F by Edman degradation, which indicated the existence of two sequences starting from the N-terminus and from Val(30). In addition, the amino acid compositions and the sequences of two peptides, Ia and Ib, obtained by reduction and *S*-carboxymethylation of M-FPI-F (Fig. 2), proved cleavage at Thr(29)-Val(30) of FPI-F by subtilisin BPN′. The incubation of FPI-F at pH 3.0 without enzyme did not generate a new sequence other than that of the N-terminus, indicating that the cleavage of the Thr(29)-Val(30) peptide bond was caused by the enzyme, but not by the acidic condition. The cleavage of the same peptide bond between Thr(29) and Val(30) was also observed using semi-alkaline protease from *A. melleus*, indicating that Thr(29)-Val(30) is also the reactive site for this fungal protease.

For the further confirmation of the reactive site, the regeneration of the Thr(29)-Val(30) peptide bond was carried out by incubation of M-FPI-F with a slight excess of subtilisin BPN′ at pH 8.0. The regenerated FPI-F from M-FPI-F was identified from the retention time on HPLC, the amino acid composition and the sequence. All results obtained according to the standard method (*16*) led us to the conclusion that Thr(29)-Val(30) is the reactive site of FPI-F, as shown in Fig. 7.

Due to the rigid structure of FPI-F, which has 55 amino acid residues containing 4 disulfide bridges, the inhibitor was hardly degraded by proteases, including thermolysin and pronase. Therefore, we devised a method to digest FPI-F to determine the location of the disulfide bridges. FPI-F was first cleaved with subtilisin BPN′ to give M-FPI-F and then the Asp(20)-Pro(21) bond in M-FPI-F was specifically cleaved by incubation in 70% formic acid at 40°C for 1 week. Thus, we could prepare doubly modified inhibitor. Thermolysin was susceptible to the doubly modified inhibitor and further fragmentation by trypsin was performed. Finally, we obtained fragments containing cystine for the determination of the locations of all four disulfide bridges in FPI-F (Fig. 3).

The location of the disulfide bridge between Cys(18)-Cys(55) was clearly confirmed by detection of PTH-cystine, which was eluted a little earlier than PTH-arginine in our PTH-amino acid analysis system, at the fourth cycle on the Edman degradation of fragments a1 and a2. The sequence of a2 indicates that a2 was derived from M-FPI-F in which the Asp(20)-Pro(21) bond had not been cleaved by 70% formic acid. On the Edman degradation of fragments b1 and b2, PTH-cystine was detected at the fourth and the seventh cycles, respectively. This result indicated that a disulfide bridge was formed between Cys(14) and Cys(27). In sequencing of fragments c1, c2, c3, c4, and c5, three PTH-amino acids were detected at the first cycle, indicating that three fragments were linked by two disulfide bridges. This complexity was caused by the Cys(35)-His(36)-Cys(37) sequence, which had not been enzymatically degraded. On the Edman degradation of c2, PTH-cystine was detected at the third and the seventh cycles indicating the locations of the disulfide bridges to be Cys(3)-Cys(35) and Cys(37)-Cys(49). This was also supported by sequence analyses of fragments c3, c4, and c5. The locations of all four disulfide bridges are illustrated in Fig. 7.

In this study, FPI-F has been characterized as a single-headed inhibitor having a reactive site composed of threonine at P_1 and valine at P_1'. The subtilisin inhibitors, which occasionally also show chymotrypsin-inhibitory activity, have methionine, leucine, or lysine at the P_1 position in the reactive site. FPI-F, having the unique P_1 residue threonine, can inhibit both subtilisin BPN′ and fungal proteases, but not chymotrypsin. Recently, a reactive site with threonine at P_1 and serine at P_1' was reported for a silkworm chymotrypsin inhibitor, a member of the Kunitz family (*19*). However, the inhibitory activity of this inhibitor toward fungal protease has not reported yet. The reactive site and the structure of FPI-F (Fig. 7) show no homology with those of Kunitz family inhibitors, in which three disulfide bridges and the reactive site are located at quite different positions from those of FPI-F.

A trypsin inhibitor of the hemolymph of solitary ascidian is composed of 55 amino acid residues with four disulfide bridges, like FPI-F (*20, 21*). However, the amino acid sequence of this inhibitor has no similarity to those of other protease inhibitors, including FPI-F. The reactive site of this inhibitor is located at Lys(16)-Met(17), which is far from the Thr(29)-Val(30) of FPI-F, and the positions of the disulfide bridges are different from those of FPI-F. Yang *et al.* purified and characterized two protease inhibitors from arrowhead plant and proposed that these inhibitors might be classified into a new protease inhibitor family (*22*). These two inhibitors also do not show any homology with FPI-F.

FPI-F is a unique inhibitor, showing no homology in amino acid sequence and no coincidence between the positions of the reactive site and the disulfide bridges, compared with those of other inhibitors. Therefore, we propose that FPI-F is a member of a new family, which we designate as the Bombyx family.

Several trypsin or chymotrypsin inhibitors from silkworm have been purified and characterized (*5, 7, 8-9, 11, 23*). Some of them have been identified as members of the Kunitz inhibitor family. While several fungal protease inhibitors have been found in silkworm hemolymph, they have not been purified yet, except for FPI-F. It is proposed that many kinds of inhibitors in hemolymph of silkworm, which does not have an immune system, play an important role in defense against the invasion of pathogenic microorganisms. The role of FPI-F in the inhibition of the growth of pathogenic fungi has been discussed elsewhere (*24*). Further investigations on the function of the inhibitor seem worthwhile.

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