Limited Proteolysis of Silkworm Antitrypsin by Several Proteinases

Takuji SASAKI,* Atuko KOHARA, Hiroshi TAKAGI
and Takaaki SHIMIDZU

Department of Food Science and Technology, School of Agriculture,
Nagoya University, Chikusa-ku, Nagoya,
Aichi 464-01, Japan

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Silkworm antitrypsin (sw-AT) isolated from larval hemolymph was limitedly digested by *Achromobacter* lysylendopeptidase, α -chymotrypsin, subtilisin BPN', subtilisin Carlsberg, papain, or *Pseudomonas* elastase. Each proteinase could cleave specific site(s) around the reactive site identified for the reaction of sw-AT and bovine trypsin. Among these proteinases, only subtilisin BPN' was inhibited by sw-AT, although weakly. By the cleavable amino acid sequence in sw-AT, it was suggested that whether or not these proteinases were inhibited by sw-AT did not solely depend on their substrate specificities. The susceptibility to the attack of proteinase should indicate that this region is exposed on the molecular surface. The amino acid sequence in the COOH-terminal region slightly away from the reactive site in sw-AT had homology with that in the corresponding region of the serine proteinase inhibitor (serpin) group.

Silkworm larval hemolymph contains several kinds of protein serine proteinase inhibitors.^{1,2)} One of them, which we named silkworm antitrypsin (sw-AT, $M_r=41,000$) can inhibit bovine trypsin. Silkworm-AT forms an equimolar complex with trypsin by an alkali-labile bond with it.³⁾ The reactive site with trypsin is near the COOH-terminus of sw-AT and the peptide bond at this site is cleaved; two fragments, 36 kDa and 5 kDa, are produced in the reaction with trypsin.³⁾ These characteristics resemble those of serpins,⁴⁾ such as α -1-proteinase inhibitor (α -1-antitrypsin),⁵⁾ antithrombin III,⁶⁾ and α -1-antichymotrypsin.⁷⁾ Most serpins have serine at their P_1' positions⁸⁾ in the reaction with proteinases, with a few exception such as α -2-antiplasmin (Met),⁹⁾ C1-inhibitor (Thr),¹⁰⁾ and mouse contrypsin (Ala).¹¹⁾ As for sw-AT, the newly produced NH_2 -terminal amino acid sequence formed by the hydrolysis of trypsin begins with valine.³⁾

On the other hand, some proteinases can cleave the α -1-proteinase inhibitor molecule at an other peptide bond near the reactive site (Met₃₅₈-Ser₃₅₉).⁵⁾ They are not inhibited by α -1-proteinase inhibitor and can inactivate it by the hydrolysis of one peptide bond not at the reactive site. In these cases, the peptide bond selectively cleaved is Ala₃₅₀-Met₃₅₁ for snake venom protease,¹²⁾ Phe₃₅₂-Leu₃₅₃ for *Staphylococcus aureus* metalloproteinase,¹³⁾ Glu₃₅₄-Ala₃₅₅ for cathepsin L,¹⁴⁾ and Pro₃₅₇-Met₃₅₈ for *Pseudomonas aeruginosa* elastase.¹⁵⁾ These facts suggest that the reactive site region of α -1-proteinase inhibitor is exposed on the surface of the molecule. Each proteinase recognizes the target site according to its cleavage specificity. Thus, some proteinases are trapped by α -1-proteinase inhibitor and others handle it only as a substrate. As mentioned above, the amino acid at P_1' position of sw-AT in the reaction with trypsin is valine. Because the true target proteinase against sw-AT has not yet

* To whom all correspondence should be addressed.

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin.

been found in silkworm hemolymph, there remains a possibility that the true reactive site is not here. Therefore, it is important to survey the amino acid sequence around the reactive site of sw-AT. For this purpose, we used several proteinases which are not inhibited or weakly inhibited by sw-AT and hydrolyze sw-AT limitedly. This way, we can find if sw-AT has a specific region near its COOH-terminus at which some peptide bonds are very susceptible to proteolytic attack and if the amino acid sequence homology between sw-AT and other serpins exists in the COOH-terminal region slightly away from the reactive site.

Materials and Methods

Materials. Bovine α -chymotrypsin (EC 3.4.21.1) was purchased from Worthington, subtilisin Carlsberg (EC 3.4.21.14), papain (EC 3.4.22.2) and *N*-trans-cinnamoylimidazole from Sigma, *Achromobacter lyticus* lysylendopeptidase (EC 3.4.21.50) from Wako, Osaka *Pseudomonas aeruginosa* elastase (EC 3.4.24.4) and subtilisin BPN' (EC 3.4.21.14) from Nagase Biochemicals, Fukushima, *N*-acetyl-L-tyrosine ethylester from the Peptide Institute, Osaka, and CM-Sephadex C-25 from Pharmacia. Reagents for sequence analysis (phenylisothiocyanate, 0.1 M Quadrol buffer, and heptafluorobutyric acid) were from Wako. Silkworm-AT was purified from silkworm larval hemolymph by the method of Sasaki and Kobayashi.²⁾

Limited reaction of sw-AT with each proteinase.

Reaction conditions of sw-AT with each proteinase are listed in Table I. Reaction products were analyzed by SDS-PAGE. For the identification of the cleaved peptide bond(s), after stopping each reaction by an inhibitor as in Table I, the pH of the solution was adjusted to pH 6.5 and it was put on a CM-Sephadex C-25 column (5 $\times$ 30 mm) equilibrated with 20 mM sodium phosphate buffer, pH 6.5. The flow-through fractions were collected and monitored by their absorbancy at 280 nm and then were dialyzed against 0.1% acetic acid for amino acid sequence analysis. In the case of *P. aeruginosa* elastase, this ion-exchange chromatography procedure was omitted because of the very small amounts used.

Inhibition of subtilisin BPN' by sw-AT.

Titration of subtilisin BPN' with sw-AT was done in 50 mM Tris-HCl buffer, pH 8.0, at 25°C. After mixing 5.3×10^{-7} M of subtilisin BPN' with sw-AT at the indicated molar ratio for 5 min at 25°C, *N*-acetyl-L-tyrosine ethylester solution (2×10^{-3} M) was added and the absorbance change at 237 nm was recorded using a Jasco UVIDEK 610C spectrophotometer. Active site titration of subtilisin BPN' was done by the method of Bender *et al.*¹⁶⁾ using *N*-trans-cinnamoylimidazole.

SDS-polyacrylamide gel electrophoresis. SDS-PAGE was done by the method of Laemmli using 10% polyacrylamide gel.¹⁷⁾ Molecular weight was estimated using standard proteins as follows: phosphorylase *b* (97,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), and soybean trypsin inhibitor (21,000).

Table I. REACTION CONDITIONS OF SW-AT AND EACH PROTEINASE

Proteinase	Proteinase concentration in reaction solution (μ M)	Buffer ^a	Molar ratio [I]/[E]	Reaction temperature (°C)	Reaction time (min)	Reaction stopper
Bovine trypsin	2.0	A	5	25	2	1 mM DFP ^b
Bovine α -chymotrypsin	1.0	A	30	25	2	1 mM DFP
<i>Achromobacter</i> lysylendopeptidase	0.25	A	80	25	2	1 mM DFP
Subtilisin BPN'	2.2	A	10	25	2	2 mM DFP
Subtilisin Carlsberg	2.1	A	10	25	2	2 mM DFP
Papain	1.6	B	20	25	1	10 mM iodoacetate
<i>Pseudomonas</i> elastase	0.02	C	1000	25	30	5 mM EDTA ^c

^a Buffer A, 50 mM Tris-HCl, pH 8.0; Buffer B, 50 mM Tris-HCl containing 5 mM cysteine and 2 mM EDTA, pH 8.0; Buffer C, 50 mM Tris-HCl containing 0.5 M NaCl, pH 7.5.

^b Diisopropylfluorophosphate.

^c Ethylenediaminetetraacetate.

Amino acid sequence analysis. Automated Edman degradation was done using a JEOL JAS-47K sequence analyzer. The coupling buffer was 0.1 M Quadrol buffer and 4 mg of polybrene was used in each run as a carrier. The conversion reaction was done manually with 20% trifluoroacetic acid at 80°C for 10 min. Phenylthiohydantoin (PTH)-amino acids were identified using reversed phase high performance liquid chromatography, by the method of Sasaki *et al.*³⁾

Results and Discussion

Treatment of sw-AT by each proteinase under the conditions listed in Table I gave the SDS-PAGE patterns shown in Fig. 1. In each case, a new protein band with a molecular mass of 36 kDa appeared. Except in the case of papain and subtilisin BPN', the intact sw-AT band (41 kDa) disappeared. Papain could not cleave intact sw-AT thoroughly under the conditions used, but further digestion was not attempted to avoid nonspecific random cleavage. Subtilisin BPN' could make a new higher molecular mass component (63 kDa) with sw-

AT. This component was stable in SDS. Other proteinases besides subtilisin BPN' and bovine trypsin could not be inhibited by sw-AT and this was supported by the absence of higher molecular weight components in SDS-PAGE (Fig. 1). In the reaction of sw-AT and bovine trypsin, a 55 kDa component corresponding to the complex of both was recognized.³⁾ Also in the reaction of sw-AT and subtilisin BPN', a 63 kDa component should be the complex of each component. Titration of subtilisin BPN' with sw-AT indicated that this proteinase activity was inhibited by sw-AT, though full inhibition of one mole of BPN' apparently required about a five-fold molar excess of sw-AT (Fig. 2). This is a reflect of the weakness of inhibition and does not mean a 1:5 complex formation. The weakness is also shown by the large amount of 36 kDa component in SDS-PAGE (Fig. 1) which represents the species of inactivated sw-AT.³⁾ A trial for isolation of the 63 kDa component by the same method as that used for isolation of the complex of trypsin

Fig. 1. SDS-PAGE Patterns of Limitedly Digested sw-AT by Each Proteinase.

Lanes 2, 6, and 11, intact sw-AT; lane 3, sw-AT+trypsin; lane 4, sw-AT+lysylendopeptidase; lane 5, sw-AT+chymotrypsin; lane 7, sw-AT+subtilisin BPN'; lane 8, sw-AT+subtilisin Carlsberg; lane 9, sw-AT+papain; lane 10, sw-AT+*Pseudomonas* elastase; lanes 1 and 12, standard proteins including phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and soybean trypsin inhibitor (21 kDa). Reaction condition with each proteinase are listed in Table I. Reaction of sw-AT with trypsin was done by the method of Sasaki *et al.*³⁾

Table II. ASSIGNED AMINO ACID SEQUENCE OBTAINED FOR sw-AT REACTED WITH EACH PROTEINASE

Proteinase used (amount analyzed)	Edman degradation cycle														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	(yield, nmol)														
Lysylendo- peptidase (4 nmol)	A	V	T	N	L	S	N	V ^a	L	K	N	G			
	1.5	2.1	0.9	2.7	1.5	— ^b	2.3		1.9	1.7	2.5	1.5			
	V	Y	V	R	P	P	I	V ^a	F	N	A	N			
	3.0	2.7	2.8	2.0	1.6	1.2	2.1		3.1	3.0	2.1	2.5			
α -Chymotrypsin (6 nmol)	A	V	T	N	L	S	N	V	L	K	N	G	N	D	N
	3.6	2.9	1.8	4.2	3.1	— ^b	3.7	2.7	2.4	1.6	4.0	1.7	3.3	2.3	3.2
	V	R	P	P	I	V	F	N	A	N	K	P	F	Y	Y
	5.0	2.5	2.0	1.8	4.4	5.3	4.5	4.0	4.1	4.8	2.4	1.0	4.4	3.4	4.3
Subtilisin Carlsberg (6 nmol)	S	N	V	L	K	V ^a	G	N ^a	D	N ^a					
	— ^b	5.1	4.6	3.0	1.8		1.9		2.8						
	V	R	P	P	I	V ^a	F	N ^a	A	N ^a					
	5.8	2.0	1.7	2.2	4.9		4.6		4.2						
Papain (6 nmol)	A	V	T	N	L	S	N	V	L	K	N ^a				
	5.2	4.2	1.9	4.9	3.9	— ^b	4.8	3.8	4.2	3.2					
	T	N	L	S	N	V ^a	L	K	N	G	N ^a				
	1.0	2.5	1.4	— ^b	3.1		1.7	1.1	1.9	1.8					
	S	S	K	V	Y	V ^a	R	P	P	I	V				
	— ^b	— ^b	3.0	2.8	2.8		1.1	0.9	1.1	2.0	2.8				
<i>P. aeruginosa</i> Elastase (18 nmol)	A	V	T	N	L	S ^a	N	V	L	K	N	G	N	D	N
	15.4	12.1	4.4	15.9	10.1	— ^b	12.3	10.3	9.4	4.6	8.2	3.2	5.6	4.6	5.1
	V	R	P	P	I	V	F	N	A	N	K	P	F	Y	Y
	8.0	6.2	1.7	2.0	5.3	6.1	6.9	7.6	5.9	5.5	2.1	0.9	3.0	2.6	3.6
	F	T	M	T	R	S ^a	S	K	V	Y	V	R	P	P	I
	9.9	3.3	4.7	2.5	1.7	— ^b	— ^b	3.5	5.9	3.4	3.7	1.7	0.9	0.7	1.8
Subtilisin BPN' (15 nmol)	A	V	T	N	L	S	N	V	L	K					
	11.1	8.4	1.6	9.9	7.2	— ^b	7.9	9.3	6.3	5.2					
	T	N	L	S	N	V ^a	L	K	N	G					
	1.9	2.4	1.7	— ^b	1.8		1.9	0.7	2.0	1.9					
	V	R	P	P	I	V ^a	F	N	A	N					
	13.9	7.2	2.3	3.8	7.1		7.7	8.3	7.8	8.6					
Bovine trypsin ^c	A	V	T	N	L	S	N	V	L	K	N	G			
	V	Y	V	R	P	P	I	V	F	N	A	N			

^a Assignment of the residue is based on the sequence obtained by other proteinase digestion.^b Not quantified^c Data from Sasaki *et al.*, *J. Biochem.*, **102**, 433 (1987).

and sw-AT³⁾ was unsuccessful. The binding between subtilisin BPN' and sw-AT may not be stable in the acylated state for a long time.

The NH₂-terminal amino acid sequence with the CM-Sephadex C-25 flow-through fraction of each proteinase digest of sw-AT was analyzed and the results were listed in Table II with the results of the cleavage of sw-AT by bovine trypsin.³⁾ The sw-AT digested by lysylendopeptidase, α -chymotrypsin, or sub-

tilisin Carlsberg produced two kinds of PTH-amino acid per Edman cycle. On the other hand, the digests by papain, *P. aeruginosa* elastase, or subtilisin BPN' gave three PTH-amino acids per cycle.

In the reaction of sw-AT and trypsin, the sw-AT molecule was cleaved in its COOH-terminal region and produced a new peptide having the sequence of Val-Tyr-Val-Arg-Pro-Pro-Ile-Val-Phe...³⁾ Though this peptide

binds noncovalently to the NH_2 -terminal fragment (36 kDa) of sw-AT, we have not yet succeeded in isolation of this peptide.³⁾ Two PTH-amino acids per Edman reaction cycle for the product by lysylendopeptidase are the same as those obtained by digestion with trypsin. Chymotrypsin can produce a peptide of its NH_2 -terminal amino acid sequence beginning with valine (Table II). This sequence coincides with that beginning from the third residue in the new sequence by lysylendopeptidase digestion. Chymotrypsin can limitedly cleave the Tyr-Val peptide bond that is two residues away from the cleavage site by lysyl-

Fig. 2. Titration of Subtilisin BPN' with sw-AT in 50 mM Tris-HCl, pH 8.0 at 25°C.

5.3×10^{-7} M of subtilisin BPN' was mixed with sw-AT at the indicated molar ratio for 5 min and 2×10^{-3} M of *N*-acetyl-L-tyrosine ethylester solution was added. The absorbance change at 237 nm was recorded. Active subtilisin BPN' was measured using *N*-trans-cinnamoyl-imidazol.

endopeptidase or trypsin. In the case of subtilisin Carlsberg digest, one of two PTH-amino acids per Edman reaction cycle are thought to originate from the same scissile bond as that by α -chymotrypsin. In the papain digest, the sequence already known, Val-Tyr-Val-Arg-Pro-Pro-Ile-Val..., is found within one of the new sequences, Ser-Ser-Lys-Val-Tyr-Val-Arg-Pro-Pro.... It is now clear that trypsin reacts with the Lys-Val peptide bond. Another new sequence should begin with the third residue of the intact NH_2 -terminus of sw-AT. In the *P. aeruginosa* elastase digest, we could identify three kinds of sequences as follows: the sequence from the original NH_2 -terminus, the sequence identical with that obtained by chymotrypsin digest, and a sequence including Ser-Ser-Lys-Val-Tyr-Val... that is found in the papain digest. The peptide beginning with phenylalanine shows the cleavage site by papain to be the Arg-Ser peptide bond. In the subtilisin BPN' digest, two main sequences, Ala-Val-Thr-Asn-Leu..., and Val-Arg-Pro-Pro-Ile... and one minor sequence, Thr-Asn-Leu-Ser-Asn..., could be traced. This indicates that subtilisin BPN' reacts with the same Tyr-Val bond as subtilisin Carlsberg or α -chymotrypsin cleaves. Though we were unsuccessful in isolating the complex of BPN' and sw-AT recognized in SDS-PAGE (Fig. 1), judging from the result obtained in the case of reaction between trypsin and sw-AT, subtilisin BPN' should also be trapped at this Tyr-Val

	①	②	③	④	
Silkworm-AT	F	T	M	T	R S S K V Y V R P P - - I V - F N A N K P F Y Y
α -1-Proteinase Inhibitor	M	F	L	E	A I P M S - I - P P E - - V K F - - N K P F V F
α -1-Anti-chymotrypsin	T	A	V	K	I T L L S A L V E T R T I V R F - - N R P F L M
Antithrombin III	T	A	V	V	I A G R S - L N P N R - - V T F K A N R P F L V
α -2-Anti-plasmin	A	T	S	I	A M S R M S L S S - - - - - F S V N R P F L F
C1-Inhibitor	A	S	A	I	S V A R T L L - - - - - V - F E V Q Q P F L P
Contrapsin	G	V	I	G	G I R K A I L - P A - - - V H F - - N R P F L F

Fig. 3. Summary of Overlapping the Sequences of sw-AT Newly Appearing upon Limited Hydrolysis with Six Proteinases and Comparison of This Result with Sequences around the Reactive Sites of Six Other Serpins. The arrows indicate cleaved sites of sw-AT by proteinases as follows: 1, *Pseudomonas* elastase; 2, papain; 3, lysylendopeptidase and trypsin; 4, α -chymotrypsin, subtilisin Carlsberg, subtilisin BPN', and *Pseudomonas* elastase. To obtain maximum homology, gaps are introduced. The vertical broken line indicates the peptide bond between the P_1 and P_1' site in each serpin.

peptide bond.

In Fig. 3, the results mentioned above are summarized with the comparison of the amino acid sequence of sw-AT with that of serpins around their reactive sites. To obtain a maximum homology, aromatic amino acids (Phe and Tyr) are aligned to get the same position in these proteins.¹⁸⁾ It is clear that sw-AT is homologous in the COOH-terminal region slightly away from the region cleavable by proteinases. Among serpins, though several cleavable sites are found in α -1-proteinase inhibitor, the P₁' position for the reaction with proteinase is almost occupied by serine. If this rule is applicable to sw-AT, there is a possibility that the reactive site with some physiologically meaningful proteinase(s) is the Arg-Ser peptide bond in this susceptible region. But, unfortunately, we have not yet identified any target proteinase(s) for sw-AT in silkworm hemolymph, and cannot conclude this position to be the true reactive site. In addition, we have no idea why bovine trypsin cannot react with sw-AT at this Arg-Ser bond. As already described above, α -1-proteinase inhibitor is cleaved at several sites by several proteinases. This should be evidence of exposure of this region on the molecular surface. In sw-AT, the corresponding region should also be exposed on the surface. Compared with the case of α -1-proteinase inhibitor, in sw-AT, there exist excess six amino acid residues between Ser and the following Pro-Pro sequence. These additional amino acids are thought to make a more distinctly exposed region than that of α -1-proteinase inhibitor and, consequently, sw-AT should be easily attacked by several proteinases in this region. The NH₂-terminus of sw-AT also seems to be on the molecular surface judging from the cleavage of Thr₃-Asn₄ and Leu₅-Ser₆ bonds by papain or subtilisin BPN' and subtilisin Carlsberg, respectively.

Silkworm-AT can make a stable complex with trypsin but not with lysylendopeptidase (Fig. 1), but the peptide bond recognized by both enzymes is the same one (Lys-Val). The difference in the stability of acylated pro-

teinases may originate from subtle differences of active site structures of both proteinases, and, in other words, the Lys-Val bond in sw-AT can recognize these proposed differences. Also with subtilisin Carlsberg and BPN', sw-AT seems to recognize the differences of active site structures of both enzymes. Both subtilisins have wide substrate specificities and are thought to have similar active site structures,¹⁹⁾ but subtilisin BPN' can be inhibited by sw-AT, though weakly, probably at the Tyr-Val bond although subtilisin Carlsberg cannot be inhibited but only cleaves the same peptide bond. These results should be used to design specific synthetic inhibitors or substrates for these proteinases.

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