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THE REACTIVE SITE OF SILKWORM HEMOLYMPH ANTICHYMOTRYPSIN IS LOCATED AT THE COOH-TERMINAL REGION OF THE MOLECULE

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SUMMARY: The complex of silkworm larval hemolymph antichymotrypsin (M_r=43,000) and C-chain of bovine α-chymotrypsin was obtained. This complex showed two NH_2-terminal amino acid sequences identical to those of intact silkworm antichymotrypsin and C-chain of α-chymotrypsin, respectively. Alkali treatment of the complex brought about its dissociation and the separated inhibitor component (M_r=36,000) had an NH_2-terminal amino acid sequence identical to that of intact silkworm antichymotrypsin. These results suggest that the reactive site of this inhibitor is located at the COOH-terminal region of the molecule and that the nature of association of this inhibitor and α-chymotrypsin is an acyl-bond.

Several protein serine proteinase inhibitors exist in human serum (1), the most well characterized inhibitor among them being α-1-proteinase inhibitor. Its amino acid sequence determined by its cDNA nucleotide sequence (2) revealed a sequence homology between it and α-1-antichymotrypsin (3) and antithrombin III (4), whose amino acid sequences were also determined by their cDNA nucleotide sequences. These three serum inhibitors resemble each other not only in their primary sequences but also in their modes of reaction with proteinases. The reactive sites of these inhibitors are located at the near end of COOH-terminal of molecules (5,6,7). Experimental results suggest the presence of acyl-bond in proteinase-serum inhibitor complexes (5,6,8).

Such serum inhibitors as described above have only been found in vertebrate serum. We have found several serine proteinase inhibitors in silkworm (*Bombyx mori*) larval hemolymph, one of which is a low molecular weight inhibitor

Abbreviations: sw-Achy, silkworm antichymotrypsin; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin.

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(M_r=7,000) and its sequence analysis revealed that it belongs to a so-called animal Kunitz type inhibitor (9). Other inhibitors include two high moleclar weight inhibitors (M_r=43,000), which lack cysteine residue and carbohydrate, one being named silkworm antichymotrypsin (sw-Achy) and another being named silkworm antitrypsin (10). Silkworm Achy can inhibit bovine α-chymotrypsin, silkworm chymotrypsin-like protease P-III and subtilisin BPN', and can also form complex with these proteinases (11). The physicochemical properties (such as molecular weight and amino acid composition) and the characteristics as proteinase inhibitor suggest that sw-Achy resembles human serum α-1-antichymotrypsin. Presented in this report is evidence that sw-Achy can make acyl-bond with bovine α-chymotrypsin and that its reactive site is located at the COOH-terminal region of the molecule.

MATERIALS AND METHODS

Silkworm Achy was prepared from hemolymph of silkworm larva (*Bombyx mori*, strain between Kinshu x Showa) of the fifth larval instar according to the procedure of Sasaki and Kobayashi (10). Alpha-chymotrypsin [E.C.3.4.21.1.](3 x crystallized) was obtained from Miles; Bio-Beads SM-2 resin from Bio-Rad; CM-52 from Whatman; and reagents for sequence analyses from Wako Pure Chem.Ltd. as sequence analysis grade.

A complex of sw-Achy and C-chain of α-chymotrypsin was formed as follows: bovine α-chymotrypsin (90% active by active site titration by 5-nitro-3H benzoxathiol dioxide, 2.4 mg, in 0.95 ml of 1 mM HCl) was mixed with 11 mg of sw-Achy (3.9 ml in 50 mM Tris-HCl, pH 8.0) for 2 min at room temperature and the reaction was stopped by adding 0.05 ml of 10 mM diisopropylfluorophosphate (Fluka). Immediately therafter, 0.4 ml of 10% $NaDodSO_4$ solution containing 0.1 M 2-mercaptoethanol was added and the mixture was boiled for 5 min. to this solution, solid urea was added to give a final concentration of 6 M, and to remove $NaDodSO_4$, the solution was incubated with 2.5 g of Bio-Beads SM-2 for 60 min at room temperature (12). After filtration, the solution was dialyzed against 0.02 M sodium phosphate buffer, pH 5.4, containing 6 M urea for 24 h at 4°C with several changes of the dialysis solution. After dialysis, the inner solution was applied to a CM-52 column (1.0 x 10 cm) equilibrated by 0.02 M sodium phosphate buffer, pH 5.4, containing 6 M urea. After washing out the passed through components, the material retained in the column was eluted by 0.2 M NaCl solution in the equilibration buffer. Finally, the obtained fractions were pooled and dialyzed exhaustively against H_2O at 4°C and lyophilized.

Alkali treatment of the complex obtained by the method mentioned above was made in 0.0, 0.02, 0.04.and 0.06 N NaOH solution containing 1% $NaDodSO_4$ at 35°C for 3 h. After incubation, the solutions were neutralized by adding equimolar HCl. To obtain the inhibitor fragment from the complex, the complex (1.2 mg) in $NaDodSO_4$ solution was treated by 0.05 N NaOH at 35°C for 3 h and then the solution was neutralized and followed by gel filtration with Sephadex G-50 superfine column (1.5 x 50 cm) equilibrated by 0.05 M sodium phosphate buffer, pH 7.0, containing 0.1% $NaDodSO_4$. The absorbancy at 230 nm was monitored and the peak fractions near the void volume were collected.

$NaDodSO_4$-PAGE was performed according to the method of Laemmli (13) using 10% acrylamide gel. As molecular weight standards, phosphorylase a (97,000), bovine serum albumin (66,000), ovalbumin (43,000), and carbonic anhydrase (29,000) were used.

Amino acid sequence analyses were carried out by JEOL JAS-47K sequence analyzer with 0.1 M Quadrol buffer, pH 9.0, as a coupling buffer and using polybrene as a carrier for each run. The obtained thiazolinone derivatives of amino acids were converted manually to PTH-amino acids by incubating with 20% trifluoroacetic acid at 80°C for 10 min. PTH-amino acids were identified by high performance liquid chromatography using TSKgel ODS-120T column (4.6 x 250 mm) by isocratic elution system (0.01 M ammonium acetate:methanol:acetonitril= 15:9:1) at 30°C. Their detection was made at 265 nm besides PTH-dehydro-Thr, which was detected at 320 nm.

RESULTS AND DISCUSSION

Shown in Fig.1 are the results of isolation of the complex of sw-Achy and C-chain of α-chymotrypsin by CM-52 as a $NaDodSO_4$-PAGE pattern. The molar ratio of sw-Achy to α-chymotrypsin in the reaction mixture was 2.9, and the electrophoretic pattern of this mixture (lane 2) gave three major protein bands whose apparent molecular weights were 51,000 (51K), 43,000 (43K), and 36,000 (36K), respectively. This pattern resembled that obtained under a similar mixing ratio without reduction. In that case, the most slowly migrated component (M_r=55,000) was assigned as complex of sw-Achy and α-chymotrypsin by fluorescence labeling and immunoblotting methods (11). In reduced case, if the complex does not dissociate by this treatment, the molecular weight of the reduced complex should be lower than that of unreduced one but higher than that of intact sw-Achy, because α-chymotrypsin is composed of three polypeptide chains connected by disulfide bonds and its active site resides in the so-called C-chain (M_r=10,000).

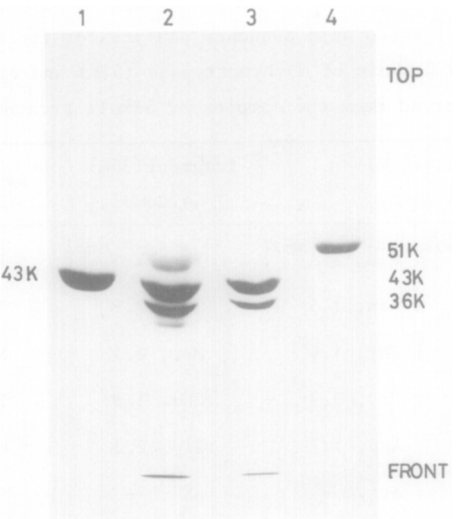

Fig.1: NaDodSO$_4$-PAGE pattern indicating the isolation of the complex of sw-Achy and C-chain of α-chymotrypsin. Silkworm Achy was mixed with α-chymotrypsin ([I]/[E]=2.9) and then the reaction mixture was reduced. Thereafter, components in the reaction mixture were fractionated by CM-52 at pH 5.4. Lane 1, intact sw-Achy, 10 μg; lane 2, reaction mixture applied on CM-52 column, 15 μg; lane 3, passed-through fraction of CM-52 column, 10 μg; lane 4, 0.2 M NaCl eluted fraction of CM-52 column, 5 μg. Molecular weights indicated are estimated by phosphorylase a (97K), bovine serum albumin (66K), ovalbumin (43K), and carbonic anhydrase (29K) as standards.

Ion-exchange chromatography by CM-52 at pH 5.4 is expected to be able to separate this complex from other components in the reaction mixture, because sw-Achy and A- and B-chain of α-chymotrypsin should be negatively charged at pH 5.4 and on the other hand, inhibitor associated with basic C-chain should be positively charged. As expected, the most slowly migrated component (51K) is clearly separated from other components (lane 4, Fig.1).

The amino acid sequence analysis of this component gave two almost equimolar PTH-amino acids in each degradation cycle. One of their sequences corresponded to the amino acid sequence to be derived successively from amino terminus of sw-Achy and the other corresponded to those from amino terminus of C-chain of α-chymotrypsin (14) (Table I). This result suggests that sw-Achy in the complex retains its intact amino terminus, that is, association of sw-Achy and α-chymotrypsin is responsible for the newly appeared terminal carboxyl

Table I

NH_2-terminal amino acid sequence analyses of the complex of sw-Achy and C-chain of α-chymotrypsin (51K) and of fragment (36K) derived from the complex by alkali treatment

cycle	complex (51K) 3.2*nmol*	fragment(36K) 4.0*nmol*	sw-Achy[a]	C-chain[b]
1	Phe, 1.7*nmol*; Ala, 2.1*nmol*	Phe, 2.1*nmol*	Phe	Ala
2	Tyr, 1.8 ; Asn, 1.5	Tyr, 2.8	Tyr	Asn
3	Met, 1.8 ; Thr, 1.6[c]	Met, 2.1	Met	Thr
4	Phe, 1.0 ; Pro, 0.9	Phe, 1.9	Phe	Pro
5	Gly, 1.6 ; Asp, 1.7	Gly, 2.3	Gly	Asp
6	X[d], - ; Arg, -[e]	X[d], -	Ser	Arg
7	Glu, 1.2 ; Leu, 0.7	Glu, 1.8	Glu	Leu
8	Phe, 1.0 ; Gln, 0.4	Phe, 1.7	Phe	Gln

a. ref.10; *b*. ref.14; *c*. identified as PTH-dehydro-Thr; *d*, not identified; *e*. not quantified.

group of sw-Achy in view of the peptide-bond hydrolyzing mechanism of α-chymotrypsin.

To confirm the character of the association present in the complex and to deduce the position of this interaction on the sw-Achy molecule, the complex was treated by alkali. Fig.2 shows the change in electrophoretic patterns after treating the complex with several concentrations of NaOH. The protein band corresponding to the complex (51K) gradually decreased in response to the increase of alkali concentration, and another component (36K) gradually increased. This finding implies that acylation occurs in the complex formation between sw-Achy and C-chain of α-chymotrypsin and, in addition, that the reactive site of sw-Achy is located at the COOH-terminal region. To verify the latter possibility, the fraction obtained by gel filtration after alkali treatment of the complex was analyzed for its amino acid sequence. This component which showed the same mobility as that of 36K component had NH_2-terminal sequence in complete agreement with that of intact sw-Achy (Table I). This result is a convincing evidence that the reactive site of sw-Achy is located at the COOH-terminal

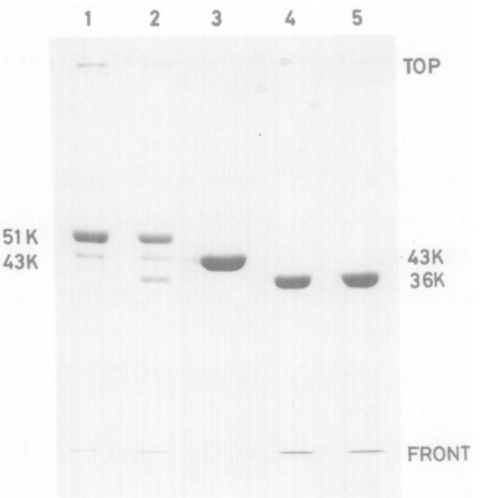

Fig.2: $NaDodSO_4$-PAGE pattern indicating the effects of alkali treatment on the complex of sw-Achy and C-chain of α-chymotrypsin. The complex (51K) was treated by NaOH solution at indicated concentration containing 1% $NaDodSO_4$ at 35°C for 3 h. Lanes 1,2,4, and 5, complex (51K, each 8 μg) were treated by NaOH in the following concentration: 0.0 N, 0.02 N, 0.04 N, and 0.06 N; lane 3, intact sw-Achy, 10 μg. Molecular weights indicated are estimated as in Fig.1.

region. The position of the reactive site is expected to be about 50 amino acid residues apart from the COOH-terminus in view of the difference in molecular weight (43K-36K). Until now, this leaving COOH-terminal peptide has not been obtained.

It is interesting to compare the forgoing results with known reactive site location and character of association with proteinases about human serum inhibitors, such as α-1-proteinase inhibitor, α-1-antichymotrypsin and antithrombin III. These three serum inhibitors which are composed of about 400 amino acid residues have their reactive sites at position about 40-50 residues apart from their COOH-terminal (2,3,4). In addition, their complexes with proteinases can be dissociated into inhibitors or their fragments and proteinases by alkali treatment (5,6,8). Their similarity to sw-Achy is obvious. This is the first case, verifying the existence of proteinse inhibitor in invertebrate which resembles that existing in vertebrate serum as for its size (about 400 amino acid residues), its association mode with proteinase (acylation) and its reac-

tive site location (COOH-terminal region). Whether the amino acid sequence homology all over the molecule exists or not is yet unknown.

REFERENCES

1. Travis,J., and Salvesen,G.S. (1983) Ann.Rev.Biochem. 52, 655-709
2. Long,G.L., Chandra,T., Woo,S.L.C., Davie,E.W., and Kurachi,K. (1984) Biochemistry 23, 4828-4837
3. Chandra,T., Stackhouse,R., Kidd,V.J., Robson,K.J.H., and Woo,S.L.C. (1983) Biochemistry 22, 5055-5061
4. Chandra,T., Stackhouse,R., Kidd,V.J., and Woo,S.L.C. (1983) Proc.Natl. Acad.Sci.U.S.A. 80, 1845-1848
5. Boswell,D.R., Jeppsson,J.-O., Brennan,S.O., and Carrell,R.W. (1983) Biochim.Biophys.Acta 744, 212-218
6. Morii,M., and Travis,J. (1983) J.Biol.Chem. 258, 12749-12752
7. Björk,I., Danielsson,A., Fenton,J.W., and Jörnvall,H. (1981) FEBS Lett. 126, 257-260
8. Fish,W.W., and Björk,I. (1979) Eur.J.Biochem. 101, 31-38
9. Sasaki,T. (1984) FEBS Lett. 168, 227-230
10. Sasaki,T., and Kobayashi,K. (1984) J.Biochem.(Tokyo) 95, 1009-1017
11. Sasaki,T., and Itou,H. (1985) Biochim.Biophys.Acta (in press)
12. Moriyama,R., Nakashima,H., Makino,S., and Koga,S. (1984) Anal.Biochem. 139, 292-297
13. Laemmli,U.K. (1970) Nature(Lond.) 227, 680-685
14. Hartley,B.S. (1964) Nature(Lond.) 201, 1284-1287