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FOUR DISULFIDE BONDS' ALLOCATION OF Na^+, K^+ -ATPASE INHIBITOR (SPAI)

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We have recently reported the primary structures of the three unique peptide inhibitors (SPAI-1, -2, and -3) against Na^+, K^+ -ATPase which contained four disulfide bridges in common (Biochem. Biophys. Res. Commun. 164, 496 (1989)). The disulfide connectivities of SPAI were determined by the combination of amino acid analyses with the direct application to a gas-phase sequencer of its proteolytic fragments. The disulfide bond was identified by detection of phenylthiohydantoin derivatives of cystine and its decomposed product dehydroalanine. The four cysteine pairs were disclosed to be Cys^{20} to Cys^{49}, Cys^{27} to Cys^{53}, Cys^{36} to Cys^{48}, and Cys^{42} to Cys^{57}, all linked by disulfide bridge formation. The allocation pattern of these disulfide bonds was the same as that recently reported for human mucous proteinase inhibitor (EMBO J. 7, 345 (1988)), though SPAI showed no proteinase inhibitory activity at all.

Last year we disclosed the amino acid sequences of three unique peptides SPAI-1, -2, and -3 which were purified from porcine duodenal extract based on the Na^+, K^+-ATPase inhibitory activity (1). It was the first time that endogenous peptide inhibitors against Na^+, K^+-ATPase had been isolated from mammalian organs. As shown in Fig. 1, SPAI-2 and -3 showed almost the same sequence except for the two amino acid substitutions at the positions 22 and 30 while SPAI-1 lacked the N-terminal twelve amino acid sequence of SPAI-2. These three peptides contained four disulfide bonds in common and are regarded as belonging to the four disulfide core proteins such as a human mucous proteinase inhibitor whose twenty amino acid residues, including eight Cys, occupy the same positions as those of SPAI (2).

It is quite interesting to know the four disulfide linkages of SPAI because SPAI does not show any proteinase inhibitory activities (1). We report herein the four disulfide connectivities of SPAI and their determination processes.

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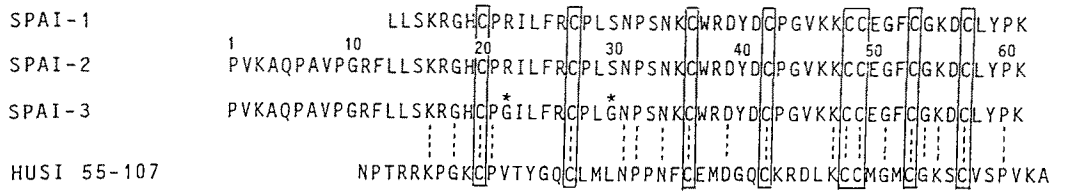

Fig. 1. The amino acid sequences of SPAIs and the second domain of human mucous proteinase inhibitor. Dotted lines indicate the common amino acids among the four peptides and the cystine residues are enclosed in boxes. *Two amino acids at the positions 22 and 30 of SPAI-3 are different from those of SPAI-1 and -2.

MATERIALS AND METHODS

Enzymatic digestion: SPAI-1 was isolated from porcine intestines as reported (1). SPAI-1 (10 nmol was digested with 10 μg of trypsin (TLCK-treated, Sigma) in 400 μl of sodium phosphate buffer pH 6.5 at 37°C for 18 hr. The reaction was stopped by adding 40 μl of 2N-HCl and then the digest was directly loaded to a HPLC ODS column (4.6 x 250 mm, YMC-Kyoto). The column was developed with the reverse phase mode by using a linear gradient of acetonitrile from 1 to 36% containing 0.1% trifluoroacetic acid (TFA) for 60 min at a flow rate of 1 ml/min. After lyophilization, the tryptic fragment T-2 was again digested with 2 μg of protease V8 (EC 3. 4. 21. 19, sequence grade, Boehringer-Mannheim) in 400 μl of 0.1 M sodium phosphate buffer pH 6.5 at room temperature for 20 hr. The protease V8 fragments (V8-1 to -4) could be separated by the same condition as that used for tryptic fragment separation.

Amino acid analysis: Each peptide was hydrolyzed with 6N-HCl containing 0.1% phenol at 110°C for 18 to 24 hr and the constituent amino acids were analyzed by the Pico-Tag method (Waters) (3). In the case of the quantitative analysis of Cys, the samples were oxidized by performic acid prior to the hydrolysis (4). About 0.5 nmol of the Cys_2-containing peptide was dissolved in performic acid reagent 100 μl which was made from 30% hydrogen peroxide 10 μl and 88% formic acid 90 μl. The reaction mixture was kept for 4 hr at 0°C and then the reagent was removed by lyophilization.

Sequence analysis: Stepwise Edman degradation was performed by a gas-phase semiautomated sequencer (Applied Biosystems, Model 470A). Disulfide bonds were not modified before sequencing and reducing reagents were omitted from direct contact with the peptide on the solid support during suquencing. Cys_2 on the sequence was identified by the detection of the phenylthiohydantoin (PTH) derivative of Cys_2 and also as the dithiothreitol (DTT) adduct of the PTH derivative of dehydroalanine (Dha) (5, 6).

RESULTS

We first tried to digest SPAI-1 with trypsin. Four tryptic fragments (T-1 to T-4) could be separated. From the amino acid analyses (Table 1) and the enzyme specificity, the structures of T-1, T-3, and T-4 could be easily deduced to be Leu-Leu-Ser-Lys, Ile-Leu-Phe-Arg, and undigested starting material SPAI-1, respectively. On the other hand, the fact that the result of the amino acid analysis of T-2 gave no integral ratio among the constituent amino acids suggested that this fraction was not homogeneous. An aliquot of the T-2 fraction was checked to show two peaks

Table 1. Amino acid compositions of all proteolytic fragments of SPAI

	T-1		T-3		V8-1		V8-2		V8-3		V8-4	
	pmol	Ratio	pmol	Ratio	pmol	Ratio	pmol	Ratio	pmol	Ratio	pmol	Ratio
Asp									222	2.01(2)	856	2.89(3)
Glu					61	1.10(1)	175	1.10(1)				
Ser	439	0.89(1)							212	1.92(2)		
Gly					66	1.21(1)	172	1.08(1)	225	2.04(2)	325	1.10(1)
His					44	0.81(1)	145	0.91(1)				
Arg			506	1.16(1)	106	1.93(2)	311	1.96(2)				
Pro					55	1.00(1)	159	1.00(1)	221	2.01(2)	617	2.08(2)
Val											247	0.83(1)
½Cys					200	3.64(4)	555	3.49(4)	201	1.83(2)	548	1.85(2)
Ile			378	0.87(1)								
Leu	925	1.87(2)	401	0.92(1)					107	0.97(1)	297	1.00(1)
Phe			435	1.00(1)					110	1.00(1)		
Lys	494	1.00(1)			48	0.88(1)			205	1.86(2)	535	1.80(2)
Tyr											529	1.78(2)

T : tryptic fragment V8 : protease V8 fragment of T-2
Cys : determined as cysteic acid

by a reverse phase HPLC using a CN column. However, taking into account the fact that all four disulfide bonds were contained in the T-2 fraction, the two fragments were not thought sufficient to estimate all the disulfide connectivities. Therefore, we chose S. aureus V8 protease for the further limited proteolysis of the T-2 farction without protease purification, because SPAI-1 contained only one glutamic acid susceptible to this enzyme. Protease V8 proteolytic fragments (V8-1 to 4) of the T-2 fraction were separated by the reverse phase HPLC using an ODS column. About one tenth of each fragment was oxidized by performic acid and then spent for the amino acid analysis. The residual materials were used for sequencing. The amino acid composition of each fragment thus obtained is shown in Table 1. V8-1 and V8-2 contained two disulfide bonds, respectively, while V8-3 and V8-4 each had only one disulfide bond. The amino acid compositions of both V8-1 and V8-2 were almost the same except that V8-1 contained one additional Lys. Edman degradation of V8-2 (800 pmol) yielded three sequences in approximately equal amounts that were assigned as follows: Gly-His-Cys-Pro-Arg, Cys-Cys-Glu, and Cys-Trp-Arg. DiPTH-Cys_2 was significantly detected only when the second half-cystine residue of a disulfide bond was split off. The direct sequencing of V8-1 gave no clear sequences due to its limited material available (200 pmol). However, V8-1 could be deduced to contain the tetrapeptide sequence Lys-Cys-Cys-Glu from the two enzyme specificities used and from the comparison of the amino acid compositions between V8-1 and V8-2.

Edman degradation of V8-3 (1.1 nmol) yielded two sequences and diPHT-Cys_2 at the third cycle while the sequencing of V8-4 (4 nmol) gave two sequences and diPHT-Cys_2 at the fourth cycle. The sequencing data and

Table 2. Results of amino acid sequence analyses and structures of protease V8 fragments

Cycle	V8-2	V8-3	V8-4
1	Gly , Cys (Cys)	Gly	Asp
2	Trp , His	Pro , Phe	Tyr
3	Glu , Arg , Cys (Cys)	Leu , Cys (Cys)	Asp , Leu
4	Pro	Gly , Ser	Tyr , Cys (Cys)
5	Arg	Asn , Lys	Pro
6		Pro	Gly , Lys
7		Ser	Val
8		Asn	Lys
9		Lys	

G H C P R (C C E / C W R) — V8-2

C P L S N P S N K / G F C G K — V8-3

D Y D C P G V K / D C L Y P K — V8-4

the assignments of the individual disulfide bridge are shown in Table 2 and the alignment of all the proteolytic fragments is summarized in Fig. 2. These results revealed that SPAI has the same disulfide linkage pattern as that of the recently determined HUSI (2).

DISCUSSION

In order to determine the four disulfide linkages, we attempted to obtain suitable proteolytic fragments of SPAI-1 without modification of the disulfide bonds so that the combination of their amino acid analyses

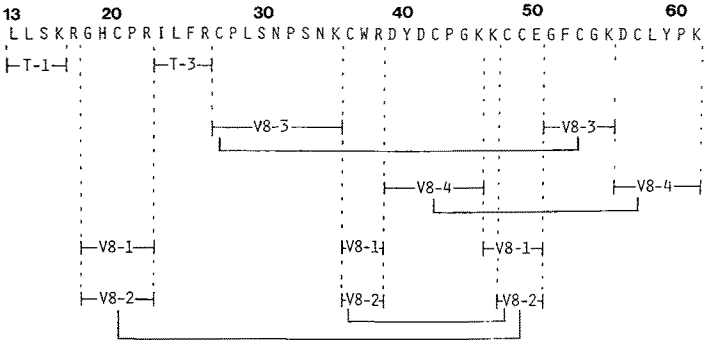

Fig. 2. Alignment of all proteolytic fragments and SS linkages of SPAI-1.
T : tryptic fragment of SPAI-1
V8 : S. aureus protease V8 fragment of T-2

with their direct sequencing results would allow us to estimate the individual disulfide bridge allocation. The first trypsin digestion split off the N-terminal tetrapeptide and the additional tetrapeptide of the sequence 23 to 26, as was expected from the enzyme specificity. Without further purification the T-2 fraction was successfully digested with protease V8 to get the four limited proteolytic faragments. The Cys_2 residue on the sequence could be easily identified by the detection of diPTH-Cys_2 as well as the DTT adduct of PTH-Dha, the latter of which was derived from the decomposition of Cys_2 (and Ser) during the Edman reaction (5). DTT was introduced later in the acidic condition at the conversion step to PTH so that the reductive fission of disulfide bond could not be expected. It is reasonably infered that both SPAI-2 and -3 have the same disulfide connective pattern as SPAI-1 because these three peptides are regarded as members of the same family.

The three dimensional structure of HUSI has been clarified by X-ray crystallography on the complex formed between bovine alpha-chymotrypsin and a recombinant HUSI (2). It is quite interesting that SPAI has no inhibitory activities on proteases, though it has not only the same amino acids in the twenty positions but also the same disulfide linkages as those of the second domain of HUSI. The primary binding site of HUSI to enzyme is suggested to be the eight residues Thr^{67} to Leu^{74} which contains the scissile bond between Leu^{72} and Met^{73} (Fig. 1) (2). This octapeptide sequence of HUSI corresponds to the sequence Ile^{23} to Ser^{30} of SPAI. However, there are no identical amino acids occupied in the common positions of both octapeptides except for the Cys residue. This fact should explain the difference in the activities of SPAI and HUSI. We are very much interested in learning whether HUSI shows the inhibitory activity on Na^+, K^+-ATPase. The physiological as well as pathological roles of SPAI have not been proposed so far, but the disclosure of the secondary structure of SPAI should assist the research for the biological diversity of the four disulfide core protins.

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