Purification and complete amino acid sequence of canine pancreatic secretory trypsin inhibitor

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Pancreatic secretory trypsin inhibitor (PSTI) was purified from canine pancreatic juice by HPLC. Canine PSTI inhibited bovine trypsin activity stoichiometrically and strongly with a dissociation constant of below 10^{-9} M. The amino acid sequence of canine PSTI was determined by conventional methods. It had one more amino acid residue at the amino-terminus than other mammalian PSTIs, i.e. human, porcine, bovine and ovine.

Trypsin inhibitor *Amino acid sequence* *HPLC* *Canine pancreatic juice* *Bovine trypsin* *Sequence homology*

1. INTRODUCTION

PSTI is produced in acinar cells of the pancreas and secreted into the pancreatic juice. The RIA system of human PSTI was established by Eddeland and Ohlsson [1] and Kitahara et al. [2,3]. Ogawa [4] reported that measurement of the serum concentration of PSTI could aid estimation of the state of acute pancreatitis. However, the human PSTI RIA system cannot be used to assay other mammalian PSTIs [3]. Establishment of the RIA system of a mammalian PSTI should enable further study of the state of acute pancreatitis with an experimental acute pancreatitis model. As a step in this direction, we purified canine PSTI from canine pancreatic juice and determined its amino acid sequence.

Abbreviations: PSTI, pancreatic secretory trypsin inhibitor; RIA, radioimmunoassay; SPase, *Staphylococcus aureus* V8 protease

2. MATERIALS AND METHODS

2.1. *Materials*

Canine pancreatic juice was collected by catheterization of the pancreatic duct after related surgical procedures. Bovine trypsin (treated with *N*-tosyl-L-phenylalanyl chloromethyl ketone) and α-chymotrypsin were purchased from Worthington. SPase was from Miles Laboratories.

2.2. *Amino acid analyses and sequence determination*

Samples were hydrolyzed according to Simpson et al. [5], at 110°C for 24 h, and amino acid analyses were performed with a Hitachi amino acid analyzer (model 835). Sequence determination was carried out as described by Kikuchi et al. [6].

2.3. *Determination of trypsin inhibitory activity*

Trypsin inhibitory activity was determined from the residual trypsin activity after mixing trypsin with inhibitor, using *N*-benzoyl-L-arginine *p*-nitroanilide as the substrate [6].

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3. RESULTS AND DISCUSSION

3.1. *Purification of canine PSTI*

Canine pancreatic juice (200 ml) was adjusted to pH 4.5 and centrifuged at 10000 × *g* for 20 min. The supernatant was subjected to gel filtration on a Sephadex G-50 column (10.5 × 101 cm) previously equilibrated with 0.1 M ammonium acetate buffer (pH 4.5). Fractions displaying trypsin inhibitory activity were pooled, adjusted to pH 7.3 and equal volumes of distilled water were added. The samples were then applied to an SP-Sephadex C-25 column (1.5 × 41 cm) equilibrated with 0.05 M ammonium acetate buffer (pH 7.3), and proteins were eluted with a linear gradient of 0–0.5 M NaCl. The PSTI fractions were pooled, dialyzed against distilled water and lyophilized. They were dissolved in a minimum volume of distilled water and subjected to reversed-phase HPLC with a column of Nucleosil C18 (Nagel, 5 μm, 4.6 × 150 mm) and 0.01 M ammonium acetate buffer (pH 4.5) as the solvent. Canine PSTI eluted with a linear gradient of 20–30% acetonitrile (fig.1) showed a single band on polyacrylamide gel electrophoresis at pH 4.3 in 7.5% gel [7]. The purified canine PSTI obtained amounted to 560 μg from 200 ml pancreatic juice with a total activity yield of 30%. We were able to purify only one form of canine PSTI with reproducible results, although 3 forms were obtained by Eddeland and Ohlsson [8]. Human PSTI also has multiple chromatographic forms [9–11], and porcine PSTI has inhibitors lacking 4 or 5 amino-terminal amino acid residues from the native PSTI [12,13]. Kikuchi et al. [6] reported that different samples of human pancreatic juice showed great fluctuation in the 4 forms of PSTI. Thus, the canine pancreatic juice that we used in this study may have only had one PSTI form.

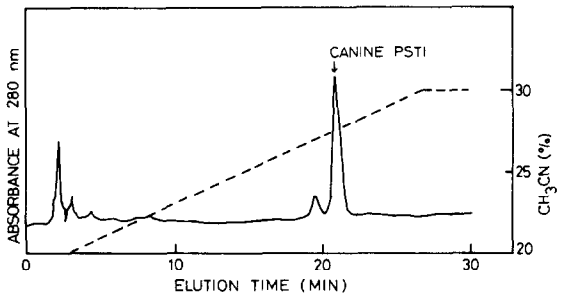

Fig.1. Purification of canine PSTI by HPLC. Canine PSTI from SP-Sephadex C-25 was applied to a Nucleosil C18 column (5 μm, 4.6 × 150 mm) equilibrated with 0.01 M ammonium acetate buffer (pH 4.5), and eluted with a linear gradient of 20–30% acetonitrile. (——) Absorbance at 280 nm, (– – –) acetonitrile concentration.

3.2. *Trypsin inhibitory activity of canine PSTI*

Canine PSTI inhibited bovine trypsin activity stoichiometrically (fig.2) and the approximate dissociation constant at pH 8 was below 10^{-9} M, when the method of Green and Work [14] was used.

3.3. *Amino acid composition and partial amino-terminal sequence of canine PSTI*

The amino acid composition of canine PSTI listed in table 1 shows 57 amino acid residues in total, which indicated canine PSTI had one amino acid residue more than other mammalian PSTIs, i.e. human, porcine, bovine and ovine [15]. The amino acid sequence of the 7 amino-terminal residues of native canine PSTI was determined by the manual Edman method to be Asn^1-Asn-Met-Leu-Gln-Arg-Gln^7.

3.4. *Amino acid sequence of canine PSTI*

Canine PSTI was reduced and *S*-carboxymethylated (RCm-) by the method of Crestfield et al. [16]. RCm-canine PSTI

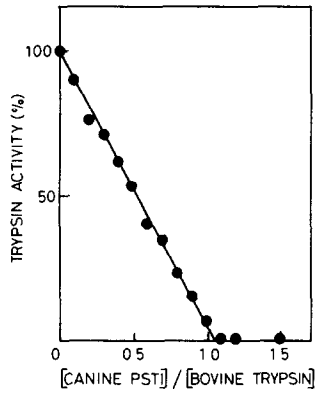

Fig.2. Trypsin inhibitory activity of canine PSTI. Trypsin concentration in the reaction mixture was 1.25×10^{-7} M.

Table 1

Amino acid composition of canine PSTI

Amino acid	Canine PSTI
Asp	9.9 (10)
Thr	1.9 (2)
Ser	2.7 (3)
Glu	6.0 (6)
Pro	1.9 (2)
Gly	3.9 (4)
Ala	2.0 (2)
1/2Cys	5.3 (2)
Val	2.0 (2)
Met	0.9 (1)
Ile	3.7 (4)
Leu	5.9 (6)
Tyr	2.0 (2)
Phe	0.0 (0)
Lys	4.8 (5)
His	0.0 (0)
Trp	0.0 (0)
Arg	2.0 (2)
Total	57

The numbers of residues are from the established sequence

(47.5 nmol) was digested with SPase and separated by HPLC as described by Kikuchi et al. [6]. Four SPase peptides (S-1 to S-4) were obtained and the amino acid sequences of S-2, S-3 and S-4 were determined as shown in fig.3. The whole amino acid sequence of S-1 was determined after further digestion by trypsin, which yielded 4 peptides (S1-T1 to S1-T4). As the amino acid sequence of S-1 corresponded to that of native canine PSTI, S-1 was the amino-terminal peptide. From the substrate specificity of SPase, S-4, which did not have glutamic acid at the carboxyl-terminus, was probably the carboxyl-terminal peptide. RCm-canine PSTI (15 nmol) was digested with trypsin (0.06 nmol) at 37°C for 4 h, the digests were separated by HPLC (as in the case of SPase digestion) and 7 peptides (T-1 to T-7) were obtained. T-1, T-2 and T-3 corresponded to S1-T1, S1-T2 and S1-T3, respectively, according to their amino acid compositions. T-4 (8 nmol) was digested with α-chymotrypsin (0.02 nmol) at 25°C for 30 min. Two peptides, T4-C1 and T4-C2, were separated using HPLC (as in the case of SPase digestion). T4-C2 covered the amino acid sequence of the S-1 carboxyl-terminal region, the whole sequence of S-2 and the S-3 amino-terminal region. Thus the sequence of the SPase peptides of RCm-canine PSTI was determined to be S-1, S-2, S-3 and S-4 in that order. These results are summarized in fig.3.

3.5. *Comparison of amino acid sequences of canine, human, porcine, bovine and ovine PSTIs*

The amino acid sequence of canine PSTI was compared with those of human, porcine, bovine and ovine [15] (fig.4). Canine PSTI had one more amino acid residue at the amino-terminus than the other mammalian PSTIs. These PSTIs had 54% homology, but it was lower near the amino-terminus than in the other positions. Anti-human PSTI antibody possesses no reactivity to canine PSTI (not shown), and the recognition site of anti-PSTI antibody to PSTI may exist around the amino-terminus.

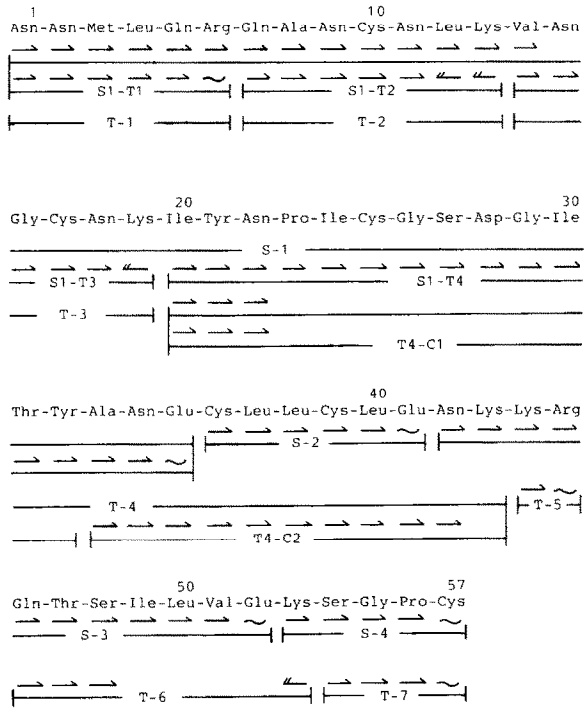

Fig.3. Amino acid sequence of canine PSTI. (—) Manual Edman degradation, (⟵) carboxypeptidase P digestion, (~) amino acid identified as a free amino acid when the residual peptide after Edman degradation was analyzed with an amino acid analyzer.

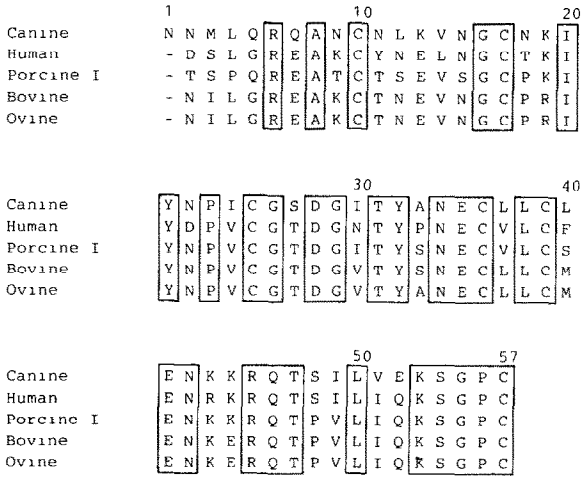

Fig.4. Comparison of amino acid sequences of canine, human, porcine, bovine and ovine PSTIs. In human PSTI, Asp^{21} and Asn^{29} differed from those reported by Greene et al. [15]; these data were reported by Kikuchi et al. [6]. Identical amino acids are framed.

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